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CHARACTERIZATION OF ALPHA-GALACTOSYLCERAMIDE AS A MUCOSAL ADJUVANT

Amy N. Courtney

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CHARACTERIZATION OF ALPHA-GALACTOSYLCERAMIDE AS A MUCOSAL ADJUVANT

by

Amy N. Courtney, BS

Approved:

Dr. K Jagannadha Sastry, PhD, Supervisory Professor

Dr. Mary K. Estes, PhD

Dr. Jeffrey K. Actor, PhD

Dr. Pramod N. Nehete, PhD

Dr. Chengming Zhu, PhD

Dr. Dapeng Zhou, MD, PhD

Approved:

Dr. George M. Stancel, PhD
Dean, The University of Texas Health Science Center at Houston
Graduate School of Biomedical Sciences

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A

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By

Amy N. Courtney, BS

Houston, Texas

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Amy N. Courtney, BS

Supervisory Professor: K. Jagannadha Sastry, PhD

Adjuvants are essential components of vaccine formulations that enhance adaptive immune responses to antigens, particularly for immunizations targeting the tolerogenic mucosal tissues, which are more biologically relevant for protective immunity against pathogens transmitted by the mucosal routes. Adjuvants possess the inherent capacity to bridge innate and adaptive immune responses through activating innate immune mediators. Here evidence is presented in support of the effectiveness of a synthetic glycolipid, alpha-Galactosylceramide (α -GalCer), as an adjuvant for mucosal immunization with peptide and protein antigens, by oral and intranasal routes, to prime antigen-specific immune responses in multiple systemic and mucosal compartments. The adjuvant activity of α -GalCer delivered by the intranasal route was manifested in terms of potent activation of NKT cells, an important innate immunity mediator, along with the activation of dendritic cells (DC) which serve as the professional antigen-presenting cells. Data from this investigation provide the first evidence for mucosal delivery as an effective means to harness the adjuvant potential of α -GalCer for priming as well as boosting cellular immune responses to co-administered immunogens. Unlike systemic administration where a single dose of α -GalCer leads to anergy of responding NKT cells and thus hinders delivery of booster immunizations, we demonstrated that administration of multiple doses of α -GalCer by the intranasal route affords repeated activation of NKT cells and the induction of broad systemic and mucosal immunity. This is specifically advantageous, and may be even essential, for vaccination regimens against mucosal pathogens such as the human immunodeficiency virus (HIV) and the human papillomavirus (HPV), where priming of durable protective immunity at the mucosal portals of pathogen entry would be highly desirable.

TABLE OF CONTENTS

	Page
Acknowledgements.....	i
Abstract.....	ii
Table of Contents.....	iii
List of Figures	vii
List of Abbreviations.....	ix
CHAPTER	
1 Background and Significance	1
1.1 Mucosal Immunity	3
1.1.1 Common mucosal immune system	4
1.1.2 Tolerance in mucosal immunization	5
1.1.3 Mucosal routes of immunization.....	6
1.2 Adjuvants	7
1.2.1 Types of adjuvants.....	8
1.2.2 Mucosal adjuvants	10
1.3 α -Galactosylceramide (α -GalCer)	13
1.3.1 α -GalCer modulates NKT cell activity.....	16
1.3.2 α -GalCer as a T-cell adjuvant	22
1.3.3 Pharmokinetics of α -GalCer	23
1.4 NKT Cell Anergy.....	24

1.4.1	Role of APCs in modulating NKT cell anergy	24
1.4.2	Role of inhibitory markers in inducing NKT cell anergy	25
1.5	Significance.....	26
2	Statement of Objectives	28
3	Materials and Methods.....	30
3.1	Animals	30
3.2	Cell lines and cell cultures.....	30
3.3	Peptides and reagents	31
3.4	Adjuvants	31
3.5	Immunizations.....	32
3.6	Analysis of antigen-specific cytolytic activity by ⁵¹ Cr release assay	33
3.7	Preparation of target cells expressing HIV env gp160	33
3.8	IFN γ ELISpot assay	34
3.9	Fluorescence labeled antibodies and flow cytometry.....	34
3.10	Intracellular cytokine staining	35
3.11	OT-1 proliferation assay	36
3.12	In vivo presentation of α -GalCer.....	36
3.13	Statistical Analysis	37
4	Results.....	38
4.1	Effectiveness of α -GalCer as an adjuvant after mucosal delivery	38
4.1.1	Comparison of different promising mucosal adjuvants	38

4.1.2	Intranasal immunization with a primary dose of α -GalCer and the HIV model peptide elicits antigen-specific immune responses	42
4.1.3	Intranasal booster immunization with α -GalCer and the HIV peptide elicits strong peptide-specific systemic and mucosal immune responses.....	45
4.1.4	Primary and booster immunizations with α -GalCer and the model HIV peptide administered by the oral route results in strong peptide-specific systemic and mucosal immune responses	49
4.1.5	Immunization with the model HIV peptide and α -GalCer by either the oral or intranasal route leads to immune recognition of the cognate HIV envelope protein.....	52
4.1.6	Booster doses of α -GalCer delivered via mucosal routes are necessary for priming antigen-specific CTL responses	55
4.2	The mechanism of mucosal adjuvanticity of α -GalCer	58
4.2.1	Intranasal immunization using α -GalCer adjuvant primes T cell responses to co-administered antigen by inducing activation of NKT cells and DC	58
4.2.2	Intranasal booster immunization employing α -GalCer adjuvant increases NKT cell activation and expansion in the lung.....	65
4.2.3	Intravenous but not intranasal immunization using α -GalCer adjuvant results in unresponsiveness of NKT cells to the booster immunization.....	72
4.2.4	CD11c+ cells and not B220+ cells present α -GalCer in the lung and lung draining lymph node after intranasal administration of α -GalCer	77
4.2.5	Evaluation of PD-1 expression on NKT cells after intranasal immunization with α -GalCer adjuvant.....	81
5	Discussion	85
6	Summary and Conclusions	91

7	Future Directions	95
7.1	Alternative α -GalCer immunization formulations.....	95
7.2	Identification of additional NKT cell anergy and NKT cell activation markers.....	98
8	References	100
9	Vita	122

LIST OF FIGURES

Figure

1.1	Structure of α -GalCer.....	15
1.2	Cross-talk between DC and NKT cells	19
1.3	Network of α -GalCer mediated activation of NKT cells.....	21
4.1.1	Effectiveness of different adjuvant candidates delivered by the intranasal route for inducing immune responses to co-administered peptide antigen	41
4.1.2	Induction of antigen-specific cellular immune responses after primary intranasal immunization with the α -GalCer adjuvant admixed with HIV peptide.....	44
4.1.3	Enhanced induction of cellular immune responses after booster intranasal immunization with α -GalCer and HIV peptide.....	48
4.1.4	Enhanced induction of cellular immune responses after repeated oral immunization with α -GalCer and HIV peptide.....	51
4.1.5	Induction of cognate protein-specific cellular immune responses after oral or intranasal immunization with α -GalCer and HIV antigen	54
4.1.6	Activation of cellular immune responses after mucosally delivered booster immunization of HIV antigen with or without the α -GalCer adjuvant	57
4.2.1	Activation and expansion of NKT cells after single intranasal administration of α - GalCer	61
4.2.2	Activation of DC after a single intranasal administration of α -GalCer	64
4.2.3	Activation and expansion of NKT cells and DC after intranasal administration of a booster dose of α -GalCer.....	69

4.2.4	Activation and expansion of NKT cells after primary but not booster intravenous administration of α -GalCer	75
4.2.5	Tissue and cell type for α -GalCer presentation after intranasal immunization	80
4.2.6	PD-1 expression on NKT cells after intravenous or intranasal administration of α -GalCer	83
6.1.1	Model of α -GalCer mediated activation of immune cells after intranasal vs. intravenous administration	94

LIST OF ABBREVIATIONS

α GalCer	alpha-Galactosylceramide
1D	1 dose
2D	2 dose
3H	Tritium
⁵¹ Cr	Chromium
AIDs	Acquired Immune Deficiency Syndrome
APC	Antigen Presenting Cell
APC	Allophycocyanin
B16	murine melanoma of C57B1/6 origin
CLN	Cervical Lymph Node
CpG	cytosine-phosphate-guanine
CpG OND	CpG oligo-deoxy-nucleotides
cpm	counts per minute
CT	Cholera Toxin
CT-2*	Mutant Cholera Toxin
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CTLL	Cytotoxic T-cell lines
DC	Dendritic Cells
DMSO	dimethyl sulfoxide
DN32.D2	NKT cell hybridoma
DNA	Deoxyribonucleic acid
dsDNA	double stranded DNA
E:T	Effector to Target Ratio
EL-4	murine T-lymphoma cell line

IgM	Immunoglobulin M
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-12R	Interleukin 12 receptor
IL-13	Interleukin 13
IL-17	Interleukin 17
IL-2	Interleukin 2
IL-4	Interleukin 4
IN	Intranasal Immunization
IP	Intraperitoneal injection
IV	Intravenous Immunization
LPS	Lipopolysaccharides
LT	heat-labile enterotoxin
MACs	Magnetic Activated Cell Sorting
MALT	Mucosal Associated Lymphoid Tissues
MdLN	mediastinal lymph nodes
MHC	Major histocompatibility complex
MLN	mesenteric lymph nodes
MPL	Monophosphoryl Lipid A
NCR	Natural Cytotoxicity Receptors
NK	Natural Killer cells
NKT	Natural Killer T cells
NV-VLP	Norwalk Virus VLP
OCH	alpha-Galactosylceramide analog
OT-1	OT-1 TCR Transgenic mice

LIST OF ABBREVIATIONS CONT.

Fab	Fragment antibody binding region
FBS	Fetal Bovine Serum
Fc	Fragment crystallizable region
FCA	Fruends Complete Adjuvant
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
GALT	Gut Associated Lymphoid Tissue
HA	hemagglutinin
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
IFN γ	Interferon gamma
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G

OVA	chicken ovalbumin
P815	Mouse lymphoblast-like mastocytoma cell line
PAMP	Pathogen Associated Molecular Patterns
PB	Pacific Blue
PBS	phosphate-buffered saline
PD-1	Programmed death 1
pDC	Plasmacytoid Dendritic Cells
PD-L1	PD-1 ligand 1
PD-L2	PD-1 ligand 2
PE	Phycoerythrin
PMA	Phorbol Myristate Acetate
RNA	Ribonucleic acid
SFC	spot forming cells
SIV	Simian Immunodeficiency Virus
ssRNA	single stranded RNA

CHAPTER 1

BACKGROUND AND SIGNIFICANCE

Vaccination is the ideal approach for sustained protection against infectious diseases and cancers linked to infectious agents. Vaccine-induced immune responses include both innate and adaptive immune cells that recognize and attack foreign pathogens based on antigen recognition, but with different mechanisms.

The innate immune system constitutes the first line of defense against invading pathogens and is comprised of multiple cell types that have the ability to rapidly recognize and respond to pathogens. The innate immune cells utilize different classes of receptors to recognize non-specific, but pathogen-related signals. These receptors can be divided into three different classes, the Toll-like receptors (TLR), nucleotide oligomerization domain-like receptors (NLRs), and activating and inhibiting NK receptors (reviewed in Turvey et al, 1). Several different innate immune cell types play important roles in the bridging of innate and adaptive immune responses, and these include dendritic cells (DC), natural killer cells (NK cells) and natural killer T cells (NKT cells). The most studied of these are the DCs, which recognize, engulf and digest pathogens by phagocytosis mediated through recognition of PAMPs and also non-specifically via macropinocytosis (2). One of the major functions of DC, in addition to this phagocytic function, is to present pathogen-specific antigens to host T cells. Because of their efficiency for this function DCs are often referred to as “nature’s adjuvant” and DCs are commonly used in vaccine formulations, either directly by injecting DCs loaded with vaccine antigens into patients, or indirectly by targeting vaccination components to DCs (3). For example, vaccination approaches against melanoma included expanding and activating DCs *ex vivo* followed by pulsing with either melanoma specific peptides or

killed Colo829 melanoma tumor cells before injecting subcutaneously into patients which was shown to induce strong antigen-specific CD4⁺ and CD8⁺ T-cell responses (4). Similarly, Nehete et al (5) reported an HIV vaccination strategy where rhesus macaques were immunized with DCs pulsed ex vivo with HIV envelope specific peptides that led to induction of virus-specific cellular immune responses and viral clearance after SHIV challenge. However, immunization strategies involving loading of DC with pathogen-specific antigens are not feasible for achieving widespread global immunization against harmful pathogens such as HIV.

Adaptive immunity is a pathogen-specific immune response resulting from exposure to the pathogen or pathogen-specific antigens/immunogens and therefore is secondary to the innate immune response in terms of kinetics of development. Nevertheless, once developed the adaptive immune response may lead to life-long immunity against the specific pathogen, an ideal goal of vaccine development strategies. The first vaccines developed against diseases such as smallpox and polio, utilized inactivated pathogens and generated protective B cell (humoral) immune responses against these pathogens. The antibodies developed against these pathogens were neutralizing in nature and sufficient to protect against infection, which suggests that a vaccine strategy that induces a strong humoral response is an effective method of protection. However, induction of strong cellular immune responses is useful for certain vaccinations, including therapeutic vaccination against cancers (6), in particular against those related to infections by viruses such as HPV (7), and chronic diseases such as those caused by HIV, hepatitis C virus, malaria and mycobacteria (8). Induction of strong CD8⁺ CTL response is also necessary for protection against certain viral pathogens such as dengue virus and HIV that are either difficult to be neutralized by antibodies or have been shown in certain instances, e.g. where antibodies are directed against mutated versions of the infectious

agent, to exhibit the phenomenon of antibody-mediated enhancement of infection and increased pathogenesis (9-19).

Most promising immunization regimens involve the administration of multiple doses of the vaccine to induce the strongest and most long lived antigen-specific immunity, either cellular, humoral or a combination of the two. Potent vaccine formulations typically include appropriate adjuvants to increase the immunogenicity of co-administered antigens and also to help overcome immune tolerance, generally through harnessing the potential of a variety of innate immune modulators. The following sections describe the general features and cell types involved in the innate and adaptive arms of the immune system with a special focus on adjuvants effective in modulating the mucosal immunity.

1.1. MUCOSAL IMMUNITY:

The mucosal immune system encompasses the mucosal tissues of the gastrointestinal tract, genital tract, and respiratory tract and contains highly specialized innate and adaptive immune cells that protect mucosal surfaces from the dangers of microbes frequently encountered through air, water and food intake as well as sexual practices. These immune cells must protect the host from potentially harmful pathogens while preventing the development of immune responses to commensal microbiota as well as harmless food and environmental antigens. These immune cells are located in various lymphoid tissues through-out the mucosa known as mucosal-associated lymphoid tissues (MALT), which consists of lymphoid compartments such as peyer's patches and the mesenteric lymph node and more diffuse aggregations of lymphoid cells found in the mucosal organs, specifically the gut and lung (20). In addition to immune cells, another player important in mucosal immunity is the secreted IgA antibody (sIgA) found in the

mucosal sites, mainly the gut, in both dimer and tetramer forms which are resistant to proteolytic degradation (21). The IgA antibody has been shown to interfere with adherence of pathogens to mucosal surfaces in addition to its ability to neutralize pathogen activity (22) and it is reported that sIgA is only induced after mucosal immunization (23).

Since a variety of pathogens (i.e. food borne pathogens, sexually-transmitted pathogens, respiratory pathogens, etc) are transmitted by mucosal routes, the induction of strong immune responses in mucosal tissues, in the form of both neutralizing antibody responses and cytotoxic T cell responses, is necessary for protection at the portals of entry. Therefore, it is believed that vaccination strategies developed for these mucosally-transmitted pathogens should elicit strong antigen-specific immune responses not only in the systemic compartment, but also in mucosal tissues. In particular, it has been shown that mucosally delivered vaccines against SIV in the non-human primate model produce strong mucosal CD8⁺ T cells and antibodies capable of blocking virus from escaping the intestinal mucosa (24, 25, 26).

1.1.1 Common mucosal immune system

It has been suggested that the effectiveness of mucosal immunization manifests in the form of the 'common mucosal immune system' wherein immune cells activated at one mucosal site are able to transmit immunity to various other mucosal sites. A recent review article of relevant literature described that human volunteers immunized by different mucosal routes exhibited antigen-specific mucosal IgA antibody responses, not only at the site of immunization, but also at distal mucosal sites, e.g. volunteers immunized by the nasal route expressed antigen-specific IgA responses in the nasal

associated mucosal tissues and the lung as well as in the genital associated mucosa (20). It is thought that mucosal specific integrins such as $\alpha 4\beta 7$ and chemokines produced by the mucosal microenvironment are involved in this process (20, 27-30). Thus, immunization regimens can capitalize on this phenomenon to prime strong antigen-specific immune responses at hard to access mucosal sites (e.g. vaginal and rectal) by delivering the vaccine formulations at relatively easy to reach mucosal sites (e.g. oral and nasal).

1.1.2 Tolerance in mucosal immunization

The mucosal immune system has evolved to recognize and respond to antigens specific to pathogens that interact with mucosal surfaces while they simultaneously have the ability to recognize and be tolerant to the antigens corresponding to various food and environmental stimulants and harmless commensal bacteria that the mucosal tissues co-exist with on a daily basis. This mucosal tolerance, reviewed in Tsuji et al 2008 (31), is due to both the presence of a variety of immunosuppressive modulators expressed within the mucosal microenvironment that prevent unwanted inflammation and antigen-specific immunosuppression resulting from the induction of anergy in immune cells and/or activation of antigen-specific regulatory T cells (Tregs). Antigen presenting cells (APC) play important roles in both of these processes because when in the immature form, the gut APCs produce regulatory cytokines (e.g. IL-10, TGF β), induce expression of several inhibitory co-stimulatory molecules (e.g. PD-L1 and PD-L2), and can present antigens to activate Tregs. However, in the mature form, gut APCs can activate both mucosal and systemic effector functions. This is best illustrated in the differences observed after mucosal immunization with either cholera toxin (CT) admixed with OVA antigen or the B subunit of CT (CT-B) admixed with OVA antigen. The former leads to

the maturation of DC and the production of IL-6 and IL-1 β by DCs inducing an immunostimulatory environment, while, mucosal immunization with CT-B and OVA does not allow for the complete maturation of DCs, which continue to produce IL-10 and TGF- β , leading to the activation of antigen specific Treg cells (32, 33, 34). Vaccine formulations administered via the mucosal routes must therefore be able to overcome these toleragenic conditions/effects by incorporating appropriate immune-stimulatory adjuvants (31).

1.1.3 Mucosal routes of immunization

Strategies for mucosal Immunization often include delivering the vaccine antigens by the nasal, oral, vaginal and rectal routes. However, the ease of vaccine administration or effectiveness to generate the desired levels of immune responses is not similar among these different mucosal routes. Vaccination by the oral route has been shown to induce immune responses in the oral tissues and the gut; however there is some concern that stomach acid and/or proteolytic enzymes may degrade immunogens (20).

Immunizations by the nasal route have been shown to effectively induce immune responses not only in the lung and nasal tissues, but also in the vaginal tissues (20, 35).

Vaccinations administered via the rectal and vaginal routes have been shown to successfully induce immune responses predominantly restricted to the gut and genital tissues respectively. (20, 36-39) This difference in cell homing is due to variable expression of chemokine receptors and adhesion molecules on lymphocytes after immunization by the various routes. For example, nasal immunization induces expression of chemokine receptor 10 (CCR10) and the integrin $\alpha_4\beta_1$ on B cells, resulting in trafficking to respiratory and uro-genital tracts, while oral immunization induces chemokines CCR9 and CCR10 as well as integrins $\alpha_4\beta_7$, and $\alpha_4\beta_1$ on B cells which

results in trafficking to the small intestine (40). Similarly, expression of chemokine receptors and integrins direct the migratory patterns of T cells after immunization by different routes. This is illustrated in the observation that T cells primed in peripheral lymphoid organs do not have the ability to migrate to mucosal tissues, since they do not express the proper mucosal homing receptors (40). Because of the potential to induce more wide-spread immune responses in addition to the ease of application, the oral and nasal routes are more popularly explored for mucosal delivery of antigens. Additionally, several recent studies have shown that mucosal immunization leads to a more protective response than systemic immunization because mucosal immunization induces an increased level of high avidity CD8+ cells at the mucosal sites (23, 41-42).

1.2 ADJUVANTS:

The working definition of an adjuvant is any substance that, when co-administered with a particular antigen, will enhance the antigen-specific immune responses. Adjuvants are particularly important for use with subunit vaccines, which are poorly immunogenic, and also in approaches for delivering vaccine formulations by the mucosal routes where it is necessary to overcome the inherent tolerance. The mechanisms underlying the effectiveness of adjuvants (i.e. adjuvanticity) varies depending on the candidate adjuvant being studied, but some common immunological processes include: A) targeting and activating APC either through TLR-dependent or TLR-independent processes, B) modulating the cytokine microenvironment to stimulate helper T cell responses, or C) inducing the recruitment of important immune modulating cell types to the site of administration and/or inducing effector function. Furthermore, the adjuvant effect can be influenced by several factors including immunization route, schedule, dosage, and

immune status of the host (43). Currently, the only adjuvant approved by FDA is alum, an aluminum salt that has proven to be safe and effective in both animals and humans. Two other adjuvants approved in Europe and currently pending approval in US include, an oil-in-water emulsion called MF59 (marketed by Novartis, Basel Switzerland), and an LPS derivative MPL (marketed by GSK Biologics, Brentford, United Kingdom), the later serves as a ligand for TLR4 (44). Since adjuvants can affect the immune responses in different ways including localization of response, induction of humoral vs cellular immunity, it is important to know how the adjuvant exerts these influences before choosing a candidate adjuvant for a specific vaccine formulation.

1.2.1 Types of Adjuvants

Oil-in-Water Emulsion Adjuvant: The most commonly used oil-in-water emulsion adjuvant is Freund's adjuvant. It is composed of an oil emulsion that either contains cell wall components from *mycobacterium tuberculosis* (Freund's complete adjuvant, FCA) or does not contain mycobacterium components (Freund's incomplete adjuvant, FIA). Both FCA and FIA are potent adjuvants; FCA promotes induction of strong antigen-specific cell-mediated immune responses while FIA is potent in aiding induction of stronger humoral immune responses to the antigen co-administered. Studies have shown that immunization with FIA and antigen induces T_H2 cytokine production (including IL-4 and IL-5), while immunization with FCA induces production of T_H1 cytokines such as IFN γ , (45-46). It has been suggested that the mycobacterium components in CFA, including CpG oligodeoxynucleotides, peptidoglycan components such as muramyl dipeptide and cell wall glycolipids such as trehalose dimycolate and lipoarabinomannan, add to the adjuvant affect of CFA by activation of PAMP receptors on DCs which skew the immune response to a T_H1 type. In the absence of these

mycobacterium components, the immune response is skewed to a T_H2 type (47). However, both FCA and FIA can induce adverse reactions at the site of injection and neither is approved for use in humans although FIA is utilized in veterinary vaccines. The mechanism of adjuvanticity of Freund's adjuvant is also due in part to the slow release of antigen formulated in the oil emulsion which is referred to as depo effect and leads to recruitment of a variety of immune cells and increased interaction with APCs (48). More recently, a new oil-in-water emulsion, MF59, has been approved for human use in many European countries, although not currently in the US. The effectiveness of MF 59 as an adjuvant is based on its capacity to trigger a cascade of immune activation, by inducing differentiation of monocytes into DC, recruiting APC to the site of administration and increasing the uptake of antigen (49).

Mineral Salt Adjuvants: Two different aluminum salts have been approved for use as adjuvants in the US for over 70 years. These aluminum salts, both commonly referred to as alum, include aluminum hydroxide and aluminum phosphate, and they have recently been shown to function by promoting antigen uptake of DC and by inducing pro-inflammatory reaction via NLR receptors at the site of administration which leads to the local recruitment of innate immune cells (1, 50). Specifically, the NLrp3 receptor is necessary for induction of both IL-1 β and IL-18, as NLrp3 deficient macrophages were unable to produce these cytokines in response to alum stimulation. Additionally, the NLrp3 receptor also appears to be necessary for the induction of antigen specific antibody response after immunization with alum, as mice deficient in NLrp3 failed to induce these responses. However, there is some variation in the literature about the effects of NLrp3 on antibody production (51-53). Alum-based adjuvants are strong and effective adjuvants; however, as described above it is known that they tend to skew immune responses towards T_H2 type cytokine production. (54-59)

TLR-Inducing Adjuvants: APCs express various TLRs and signaling through TLR-ligation can lead to activation of immune responses. Different TLRs recognize a variety of ligands that contain pathogen associated molecular patterns (PAMPs). For example, TLR4 recognizes LPS of gram negative *E. coli* as the ligand while TLR3 recognizes dsRNA, typical of certain viruses. The ability of various TLR ligands to activate APC and subsequently stimulate immune responses makes them attractive as adjuvants. Some of the more studied TLR-activating adjuvants include CpG, a type of unmethylated bacterial DNA that interacts with TLR9, and imiquimod a TLR7/8 ligand, which have been shown to activate plasmacytoid DC (pDCs) to induce antigen-specific CD4⁺ and CD8⁺ T cell responses (60)

1.2.2 Mucosal Adjuvants

Certain adjuvants are better suited for mucosal administration than others. For example, oil-in-water emulsions may not be easily delivered by the nasal immunization route, however certain TLR-inducing adjuvants could. In addition to the ease of application, mucosally-delivered adjuvants must be potent enough to overcome the tolerogenic mucosal environment. Effective mucosal adjuvants include bacterial DNA (CpG), virus-like particles (VLPs), cytokine/chemokines, and bacterial toxins such as cholera toxin (CT) encoded by *Vibrio cholerae* and heat-labile enterotoxin (LT) encoded by *E. coli*.

Bacterial Toxin Adjuvants: The most widely studied bacterial toxins include CT and LT which function as potent adjuvants, and are thought to work by activating DC (61-62). For example, Anjuere et al (62) showed that oral immunization with CT led to the activation and migration of gut DC to the MLN, which when isolated could activate

antigen-specific T cells in vitro to produce T_H2 cytokines. These toxin adjuvants have been shown to be effective in inducing antigen specific cellular and humoral immunity when delivered by the mucosal routes (e.g. nasal and oral) however their toxic effects are too harmful for use in humans. In addition, it has been shown that nasal administration of bacterial toxins leads to migration of the co-administered antigens to the olfactory neuroepithelium (63). To avoid the toxic effects, many studies have been performed on different mutant forms and subunits of these toxins, and many of the less toxic forms, including a 2 codon mutation of cholera toxin called CT-2*, have also proven to be strong adjuvants when delivered mucosally (35, 64-66). For example, Manuri et al (35) has shown that intranasal immunization with CT-2* and HPV antigens induce T cell immune responses that protected against HPV tumor challenge in mice.

CpG: Bacterial DNA contains un-methylated dinucleotides, referred to as CpG, which can interact with TLR9 to activate APCs and other immune cell types. A variety of synthetic oligo-deoxy-nucleotides rich in un-methylated CpG motifs (also referred in the literature as CpG-ODN) have been designed and tested as adjuvants successfully after administration by both systemic and mucosal (oral and nasal) routes to induce antigen-specific immunity. It has been reported that the CpG-ODN generally skew the adaptive immune response towards more potent T_H1 cytokine production. For example, Huang CF et al (67) immunized neo-natal mice by the sublingual route with either CT mixed with OVA or CpG mixed with OVA and found that the mice immunized with CpG has a strong T_H1 response whereas CT induced a strong $TH2$ response (67). However, it is difficult to predict human immune responses to vaccines formulated with CpG-ODN, due to differences in the host cell types for TLR9 expression and distribution, and differences in the types of CpG antigens need to stimulate TLR9 between humans and mice (64, 68-69).

Virus-like Particles (VLPs): VLPs are structures composed of the outer 'shell' proteins of viruses (capsid proteins) that contain no nucleic acid, and are therefore non-replicating. VLPs are thought to be the appropriate size for uptake by DC for activation and are suitable for a variety of administration routes to exhibit adjuvant activity, although the exact mechanism for any particular VLP to be potentially effective as an adjuvant is not fully understood. VLPs as immunogens or vaccine candidates have been shown to induce both humoral and cellular immune responses. For example, intranasal immunization of mice with an HIV specific VLP was shown to be effective in the production of both systemic and mucosal HIV specific IgG and IgA responses and increased antigen-specific CTL activity (70). VLPs have been created from HIV, SIV, HPV, Norwalk Virus as well as many other viruses, and the currently approved HPV vaccine, Gardasil (Merck, Whitehouse Station, New Jersey) is composed of HPV specific VLPs, indicating that they may be safe for human use. To date, most studies with VLPs have been utilized to induce self immunity, not immunity against a co-administered antigen (71).

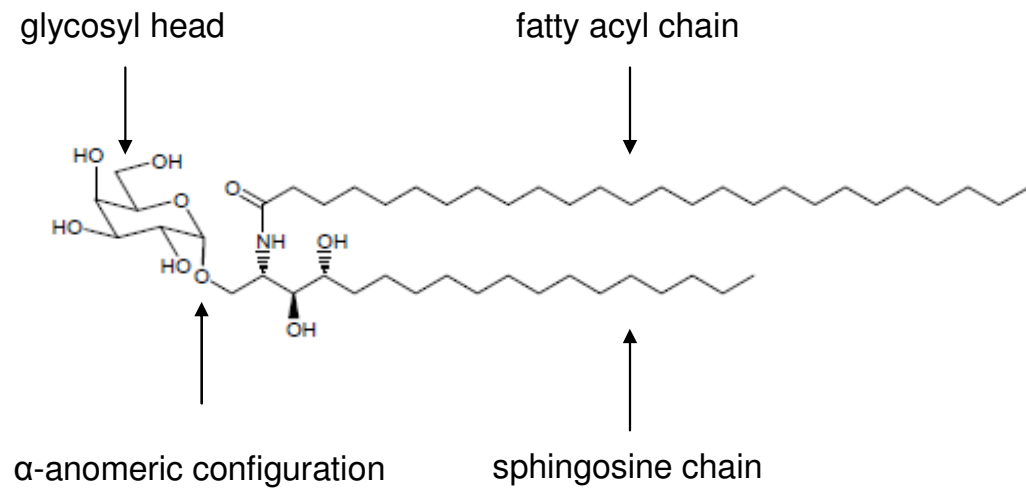
1.3 α -GALACTOSYLCERAMIDE (α -GalCer):

The glycolipid α -GalCer was originally isolated from marine sponge extracts and was the first ligand identified that when presented by dendritic cells and other APCs in the context of CD1d molecules is capable of activating NKT cells, an important mediator of innate immunity. The Japanese biotech company Kirin Pharmaceuticals was the first to identify and test α -GalCer. Several groups have explored the structural interactions between α -GalCer, the CD1d molecule on APCs and the T cell receptor (TCR) on NKT

cells. It has been shown that both the sphingosine chain and the acyl chain of α -GalCer interact tightly with the CD1d molecule (72) while the glycosyl head group is left exposed to interact with the TCR on the NKT cells (73). It was shown that the α -anomeric configuration of the inner sugar of α -GalCer was important for NKT cell stimulation and the lengths of the fatty acyl chain and sphingosine base were also important, with the shorter sphingosines lacking the ability to stimulate NKT cells, perhaps due to weaker interactions which lead to faster disengagement from CD1d (72, 74). The optimal length of these chains is a fatty acyl group with 26 carbons and a sphingosine base with 18 carbons (Fig. 1.1). These chains are hypothesized to interact hydrophobically with the CD1d molecule on APCs (74). It was reported that administration of the glycolipid to mice protected against B16 melanoma challenge, and analyses of the mechanism for protection lead to the identification of invariant NKT cells as the target for the stimulation by α -GalCer (75-76).

Fig. 1.1: Structure of α -Galactosylceramide (α -GalCer). α -GalCer is a glycosphingolipid that consists of a sphingosine base, fatty acyl chain and an α -anomeric sugar moiety. This figure is adapted from α -GalCer Data Sheet from Funakoshi/DiagnoCine (Hackensack, NJ).

Fig. 1.1: Structure of α -Galactosylceramide (α -GalCer).



1.3.1 α -GalCer modulates NKT cell activity

It is well recognized that α -GalCer administered by systemic injection leads to presentation by the CD1d molecule on APC to NKT cells, and in combination with interactions between co-stimulatory molecules such as OX40 on NKT cells and OX40L on APC results in the activation of NKT cells (77-78). Activation of NKT cells is followed by up-regulation of CD40L, and production of various T_H1 and T_H2 cytokines (Fig. 1.2). Early on in NKT cell stimulation, the T_H2 cytokine IL-4 is produced in robust amounts peaking at approximately 4 hours after stimulation, while the production of T_H1 cytokine IFN γ is more prolonged peaking at approximately 24 hours after stimulation. The CD40L on the NKT cells can interact with CD40 expressed on DCs and induce the activation of DC that include up-regulation of other co-stimulatory markers CD80 and CD86 and the expression of the cytokine IL-12. The IL-12 produced by DC can in turn enhance activation of NKT cells. The large amounts of IFN γ produced by NKT cells can lead to the trans-activation of NK cells, CD4 $^+$ T cells and B cells. Additionally, activated NK cells produce more IFN γ , resulting in more trans-activation of the immune cells. The T_H2 cytokines produced by the activated NKT cells, including IL-4 and IL-13, can also activate both CD4 $^+$ T cells and B cells. Finally, the activated DC, in addition to exhibiting increased expression of co-stimulatory molecules also exhibits enhanced antigen presentation, which can lead to the induction of adaptive T cell responses (75, 79-85) to the co-administered antigens (Fig. 1.3). A similar sequence of events was observed after the injection of α -GalCer pulsed DC, with more sustained and efficient responses in terms of IFN γ production and T cell stimulation (86-87). Between 8 and 24 hours after α -GalCer stimulation, the NKT cells down-regulate the cell surface TCR, and then begin to rapidly multiply, reaching peak expansion at day 3 post α -GalCer administration. The

NKT cell population is then constricted via apoptosis and the population reaches homeostatic levels between days 7 and 10 post administration (88-89).

Fig. 1.2: Cross-talk between DC and NKT cells. Depiction of the signaling mechanisms operating for NKT cell activation that in turn leads to activation of DC after systemic administration of α -GalCer. The glycolipid (α -GalCer) is presented by the CD1d molecule of antigen presenting cells (e.g. DC) to the T cell receptor (TCR) on the NKT cells (i). The interaction between CD1d (on APC) and the TCR (on NKT cells) is followed shortly afterwards by activation of NKT cells and expression of cytokines (ii). Next, DC become activated through surface marker interactions with the NKT cell, (e.g. CD40:CD40L interactions) and also cytokines produced by the NKT cells (iii). The DC activation leads to expression and secretion of the cytokine IL-12 and up-regulation of co-stimulatory markers such as CD80 and CD86. The IL-12 interacts with the IL-12R located on the NKT cell surface (iv) and this leads to further activation of the NKT cell, inducing a positive feedback loop. In addition to these events, further interactions between CD28 on the NKT cell and CD80/86 on the DC and OX40 on the NKT cell and OX40L on the DC can also lead to increased activation of these cell types. This figure is adapted from Zhou D. 2007 (77).

Fig. 1.2: Cross-talk between DC and NKT cells.

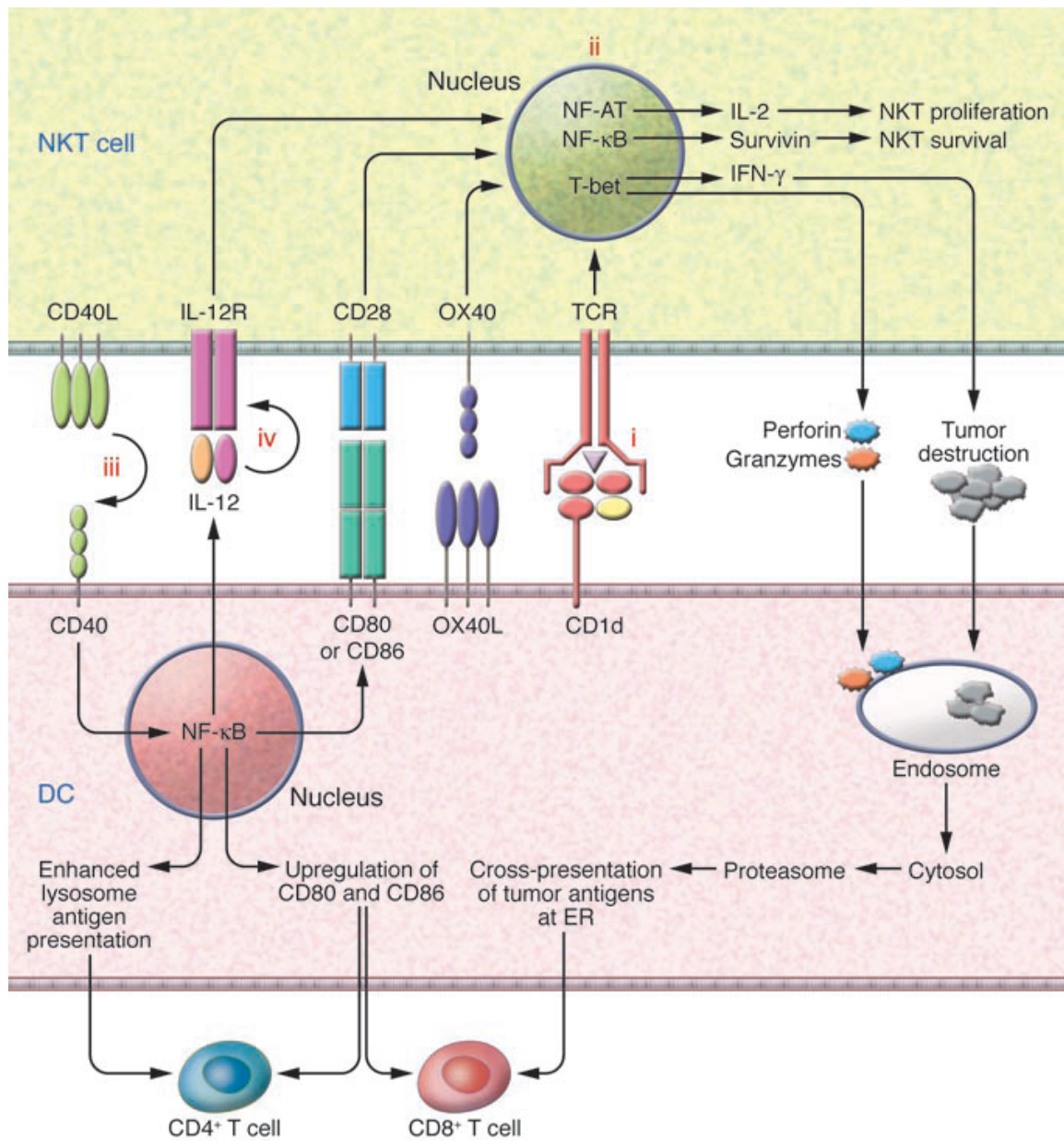


Fig. 1.3: Network of α -GalCer mediated signals in the activation of NKT cells.

Presentation of α -GalCer on the CD1d molecule of DC to the V α 14J α 18 TCR on NKT cells leads to the activation of NKT cells which induces the production of the cytokines IL-4, IL-13 and IFN γ . Interactions between the CD40L on NKT cells with the CD40 on DC along with stimulation by IFN γ and IL-4 induce activation of the DC and leads to the production of IL-12. Furthermore, IFN γ production leads to the activation of other cell types including CD8 $^{+}$ T cells, NK cells and CD4 $^{+}$ T cells. Production of IL-4 and IL-13 by NKT cells leads to further activation of CD4 $^{+}$ T cells and B cells. This figure is adapted from Bendelac A, et al 2007 (75).

1.3.2 α -GalCer as a T-cell Adjuvant

Medical utility of α -GalCer was first realized based on its ability to induce regression of B16 tumors in mice (76). Since this initial discovery, a number of studies explored the anti-tumor effects of α -GalCer. It has been found that, in addition to the antigen non-specific tumor lysis mediated by NKT cells and the activated NK cells, α -GalCer administration also stimulates antigen-specific T-cells, which can be exploited to present tumor-specific antigens, either by co-administration of tumor antigens or irradiated tumor cells along with α -GalCer (90-91). For example, Nakagawa et al (92) showed that treatment with α -GalCer alone beginning on Day 1 after inoculation of mice with an adenocarcinoma tumor cell line (Colon26) that developed liver metastases was sufficient to inhibit tumor growth in the liver (92). It was also discovered that injection of DC pulsed ex vivo with α -GalCer increases the anti-tumor effects of α -GalCer, as shown by Fujii et al (86) who injected α -GalCer pulsed DCs into mice the same day they were inoculated with B16 melanoma tumors and found greatly reduced lung metastases. Although α -GalCer treatment has proven to be effective for tumor regression in mice, similar results have not been obtained in human trials to date. The α -GalCer-pulsed DCs have been delivered to human cancer patients sub-mucosally or intravenously, and the expansion and activation of NKT cells, including cytokine production, has been observed, but without clinical benefit (87, 93-94). In addition to cancer therapies, α -GalCer has also been tested as a vaccine adjuvant in mouse models for protection against various pathogens including influenza, HIV and Malaria (83, 95-96). α -GalCer has been shown to activate both cellular and humoral immunity towards these pathogens. For example, Huang et al (67) recently reported injection of mice intramuscularly with an HIV DNA vaccine and α -GalCer, and found increased antigen-specific CD8⁺ and CD4⁺ T cell immune responses. In addition, immunization with α -

GalCer and the DNA vaccine induced a 10 fold higher level of anti-HIV gag IgG antibody response than immunization of mice with the DNA vaccine in the absence of the adjuvant (97).

1.3.3 Pharmokinetics of α -GalCer

α -GalCer has been administered to human cancer patients with refractory solid tumors and studied to determine the safety of the drug (Giaccone G et al, 98). In this study it has been shown that i.v. administration of α -GalCer weekly for 3 weeks at various dose ranges spanning almost a 100-fold range (with the lowest concentration at $50\mu\text{g}/\text{m}^2$) did not induce any dose-limiting toxicity. Also, no maximum tolerance dose was observed in this 100-fold range and α -GalCer did not accumulate in the serum with multiple doses. Additionally, urine excretion was below the limit of detection and α -GalCer was detected in serum at day 1 post immunization, with the detection level decreasing each day after immunization. Only one patient out of 24 experienced any side effects which were limited to fever and shivering and these side effects were abated after treatment with analgesics. Patients were also monitored for any immunological effects of α -GalCer administration, and increases in several cytokines were observed, including IFN γ , IL-12, GM-CSF and TNF α . Overall this study suggests that α -GalCer is safe to use in humans (98). Two other safety studies have been performed to determine the safety of injecting free α -GalCer. These studies have focused on hepatitis patients and both have found that α -GalCer is safe for human use (99-100). Additionally, several human clinical trials have been performed utilizing DCs pulsed with α -GalCer, and these trials have also illustrated that α -GalCer is safe for human use (87, 93).

1.4 NKT CELL ANERGY:

Despite the impressive safety profile and encouraging adjuvant potential, one concern for clinical utility is based on reports by several groups that systemic administration of α -GalCer (e.g. by intravenous and intra-peritoneal routes) resulted in the induction of NKT cell anergy in the murine model. It has been observed that, after the initial activation, NKT cells become functionally unresponsive to re-stimulation with α -GalCer, such that they do not produce cytokines, proliferate or trans-activate other immune cells. This unresponsive or anergic phenotype of the NKT cells was observed to persist for as long as 60 days after the initial α -GalCer administration. However, the NKT cells can be rescued from the anergic state when co-stimulated in vitro with either the cytokine IL-2 or cultured with PMA and ionomycin, which is similar to the IL-2 and PMA/ionomycin induced re-stimulation that has been observed in vitro in some instances of T cell anergy (101-102).

1.4.1 Role of APCs in modulating NKT cell anergy

Although injection of α -GalCer induces NKT cell anergy, Fujii et al (86) has shown that α -GalCer pulsed DCs can be injected for multiple doses without inducing the unresponsive phenotype to the NKT cells. It is hypothesized that anergy to NKT cells results when the free α -GalCer is presented by APC other than DC. Subsequently, Parekh et al (101) performed similar experiments where injection of α -GalCer pulsed B cells to mice resulted in NKT cell anergy. Additionally, Bezbradica et al (103) determined that B cells had a suppressive effect on NKT cell functions in vivo by showing that when mice were depleted of B cells, the α -GalCer mediated NKT cell activation as measured by cytokine production was 3 to 5 fold higher than that observed

in wild-type mice. Therefore, it has been suggested that α -GalCer presentation should be specifically targeted to DC to avoid inducing NKT cell anergy. It is particularly important to avoid anergy to NKT cells when developing α -GalCer based vaccines/therapeutics that may require repeated dosing.

1.4.2 Role of inhibitory markers in inducing NKT cell anergy

In addition to the role of specific APC types, certain inhibitory markers have also been proposed for their roles in inducing anergy to NKT cells. The expression of several known inhibitory receptors on NKT cells was assessed after α -GalCer stimulation in vivo and this included determining the expression of Ly49D, Ly49F, CTLA-4 and CD154 on both NKT cells and T cells, which were found to be unaffected by α -GalCer administration (102). However recently, several groups have analyzed the role of the inhibitory marker programmed death-1 (PD-1) for NKT cell anergy. After α -GalCer mediated stimulation PD-1 was observed to rapidly increase on NKT cells and the levels remain elevated for at least 2 months post-stimulation (104-105). The PD-1 molecule has been studied predominately as a marker on T cells, where its interaction with the ligands PD-L1 or PD-L2 expressed on DC was shown to lead to T cell exhaustion, in terms of reduced proliferation and cytokine production in addition to decreased cytolytic function. This PD-1 mediated T cell exhaustion phenotype has been found in chronically HIV infected patients, where blockage of the PD-1/PD-L1 interaction has been shown to restore the functional capacity to the exhausted T cells (106-108). Similarly, the blockage of the murine PD-1/PD-L1 interaction after α -GalCer stimulation by injection of antibodies specific for either PD-1 or PD-L1 every 3 to 4 days post α -GalCer stimulation led to the prevention NKT cell anergy, as measured by the re-stimulation of NKT cells either in vitro or in vivo (105). Additionally, PD-1 deficient mice can be immunized

repeatedly with α -GalCer by the i.p. route without inducing NKT cell anergy, as measured by the repeated activation and expansion of NKT cells (104-105). These studies suggest that the PD-1/PD-L1 interaction plays a crucial role in the induction of NKT anergy.

1.5 SIGNIFICANCE

There is a need for the development of new mucosal adjuvants that can safely be delivered by mucosal routes, preferably multiple doses at different times. Currently, there are only 5 approved mucosal vaccines to such disease as polio, cholera, typhoid, rotavirus and influenza. Of these, the polio, typhoid, rotavirus and influenza vaccines are comprised of live attenuated pathogens. However, immunization with live attenuated pathogens may not be an acceptable strategy for certain viral pathogens, including HIV (40). Therefore, subunit vaccinations that can be effectively delivered by the mucosal route may be a practical alternative, but such vaccinations require the inclusion of safe and effective adjuvants for efficacy. The most effective adjuvants studied to date include bacterial toxins, which are not safe for human use, and TLR stimulators, which are safe for human use. Investigations for this dissertation concentrated on α -GalCer, which is a strong and effective adjuvant when delivered by parenteral routes (84, 86, 90). Recently it has been shown to be safe for delivery by intranasal routes, with no redirection to the olfactory tissues, a problem encountered with certain adjuvants based on bacterial toxins (95). Reports also described parenteral administration of α -GalCer to be safe in humans in several clinical trials (87, 109). In addition to safety, α -GalCer has the ability to activate multiple arms of the innate and adaptive immune systems, including activation of B and T cells, NKT cells, NK cells and DC (75). Activation of multiple arms of immunity may be necessary for protection against certain mucosal pathogens such as HIV, herpes virus and mycobacterium (23). In particular, an adjuvant that can effectively

activate strong antigen-specific B and T cell immune responses is particularly attractive for HIV vaccinations. Additionally, NK cells have been shown to play a role in HIV infection, therefore activation of both NK and NKT cells may also aid in clearance of the pathogen. The present study for this dissertation constitutes systematic analyses of the effectiveness of α -GalCer as an adjuvant for peptide and protein antigens delivered by mucosal routes (nasal and oral) in order to induce cellular immune responses to the co-administered antigens in both the systemic and mucosal tissues. Data from this investigation provide the first evidence for mucosal delivery as an effective means to harness the adjuvant potential of α -GalCer for priming as well as boosting cellular immune responses to co-administered immunogens. Unlike systemic administration where a single dose of α -GalCer leads to anergy of responding NKT cells and thus hinders delivery of booster immunizations, we demonstrated that administration of multiple doses of α -GalCer by the intranasal route affords repeated activation of NKT cells and the induction of broad systemic and mucosal immunity without the induction of NKT cell anergy. These results bode well for formulating vaccines against mucosal pathogens where induction of mucosal immunity along with systemic immunity is of importance, and vaccine formulations need to be delivered multiple times for sustained pathogen-specific immunity. Thus, the major significance of the investigation lies in the realization that α -GalCer, a non-toxic synthetic lipid can be effectively used for mucosal vaccine formulations. In addition, this investigation demonstrates the importance of mucosal delivery to overcome the inherent problem of anergy development to NKT cells by the parenteral routes of immunization.

CHAPTER 2

STATEMENT OF OBJECTIVES

Adjuvants are reagents utilized in vaccination approaches to enhance adaptive immune responses to antigens, particularly for immunizations targeting the tolerogenic mucosal tissues, which are more biologically relevant for induction of protective immunity against mucosally-transmitted pathogens. One important mechanistic aspect of the effectiveness of adjuvants is their inherent capacity to bridge innate and adaptive immune responses through activating innate immune mediators. Availability of strong mucosal adjuvants that are safe for large-scale human use is an unmet need, specifically for vaccination approaches against human pathogens such as HIV and HPV that are transmitted predominantly by the mucosal routes. Alpha-galactosylceramide (α -GalCer) is a synthetic glycolipid, which is shown to be capable, when delivered by systemic routes, of activating natural killer T (NKT) cells, important mediators of innate immunity. **In the present investigation, I tested the hypothesis that α -GalCer will be a suitable mucosal adjuvant to induce both systemic and mucosal antigen-specific immune responses.** I obtained evidence in support of the effectiveness of α -GalCer as a mucosal adjuvant for priming as well as boosting of mucosal and systemic immune responses to co-administered immunogens by either the oral or nasal route. Additionally, I found that both the priming and booster doses of α -GalCer delivered by the intranasal route induce activation of the NKT cells and DC, unlike after systemic immunization of α -GalCer which leads to NKT cell anergy. The following specific aims were developed to address my central hypothesis that α -GalCer is a potent mucosal adjuvant and to understand the underlying mechanism.

Specific Aims

Aim 1: Determine the effectiveness of α -GalCer as an adjuvant to prime antigen-specific systemic and mucosal immunity after mucosal delivery.

Mice were immunized by the intranasal or oral routes with a combination of α -GalCer and the HIV model peptide R15K (RIQRGPGRAFVTIGK) or ovalbumin (OVA) protein and cellular immune responses were determined using the standard chromium release assay to measure antigen-specific CTL activity and the IFN γ ELISpot assay to enumerate the numbers of antigen-specific IFN γ producing cells in various systemic and mucosal tissues.

Aim 2: Determine the mechanism of mucosal adjuvanticity of α -GalCer.

Mice were immunized with a combination of α -GalCer and the chicken ovalbumin (OVA) protein and sacrificed at various time-points post-immunization to determine the response of various immune cells, such as the NKT cells, DC and T-cells in multiple systemic and mucosal tissues. NKT cell activation and expansion were assessed by flow cytometry and the utilization of the α -GalCer-loaded CD1d tetramer and DC activation was assessed via CD86 expression. Immune cell responses were measured after both a primary and booster dose of α -GalCer and OVA delivered by the intranasal route and were compared to those after systemic immunization by the intravenous route. Additionally, the tissue of origin and phenotype of α -GalCer presenting cells after mucosal immunization was assessed by co-culturing cells isolated from different mucosal and systemic tissues of mice immunized by the intranasal route with α -GalCer/OVA or OVA alone. Finally, NKT cells from mice immunized with α -GalCer and OVA by the intranasal and intravenous routes were comparatively analyzed for the expression of markers of functional exhaustion (e.g. PD-1).

CHAPTER 3

MATERIALS AND METHODS

3.1. ANIMALS

Female Balb/C and C57Bl/6 mice 6-10 weeks of age were purchased from the National Cancer Institute (Bethesda, MD) and maintained in a specific pathogen-free environment at The University of Texas M.D. Anderson Cancer Center. All procedures on the animals were carried out in accordance with institutionally approved protocols. The animal facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animals Care International and the studies were conducted according to National Institute of Health Guidelines on the care and use of Laboratory Animals.

3.2. CELL LINES AND CELL CULTURES

The cell lines EL-4 (C57BL/6, H-2b, Thymoma) and P815 (DBA/2, H-2^d, Mastocytoma) were maintained in RPMI complete media (CM) supplemented with 10% heat inactivated FBS, 50 U/mL of penicillin-streptomycin and 50µg/mL gentamycin. The DN32.D2 hybridoma NKT cells and IL2-dependent CTLL cells were maintained in a 50:50 mixture of RPMI and EHAA (Invitrogen, Carlsbad, CA) media supplemented with 10% heat inactivated FBS, 100U/mL of penicillin-streptomycin, 25µg/mL gentamycin and 55µM β-Mercaptoethanol.

3.3. PEPTIDES AND REAGENTS

Synthetic peptides corresponding to the CTL epitope of chicken ovalbumin (SIINFEKL) and known CTL epitopes from the V3 region of the HIV-1 envelope protein gp120 from HIV-1 IIIB including R15K, RIQRGPGRAFVTIGK (110) and the specific H-2^d epitope peptide R10I found within the R15K sequence, RGPGRAFVTI (111) were utilized in immunization procedures as well as for cellular assays. All the peptides were synthesized by the solid-phase method of Merrifield (112) either on a modified Vega250 automatic peptide synthesizer (Vega Biochemicals, Tucson, Arizona) or by the 'Bag' method as described by Houghten (113). The peptides were >90% pure as determined by high-pressure liquid chromatography (HPLC) and mass spectrometry analyses. OVA protein was purchased from Sigma (St Louis, MO) and re-suspended in 1x phosphate-buffered saline (PBS; Gibco, Carlsbad, CA).

3.4. ADJUVANTS

α -GalCer was purchased from either Alexis Biochemicals (San Diego, CA) or Diagnocine LLC (Hackensack, NJ) and dissolved in dimethyl sulfoxide (DMSO; Sigma, St Louis, MO). CT-2*, a mutant of the cholera toxin, was obtained from Dr. Ashok Chopra at the University of Texas Medical Branch (Galveston, TX). The CT-2* protein was purified by sodium hexametaphosphate precipitation, affinity purification on a galactose column and sephadex G75 gel filtration chromatography as described by Boesman-Finkelstein (114) and Van (115). The Norwalk Virus-Like Particles (NV-VLP) were obtained from Dr. Mary Estes at the Baylor College of Medicine (Houston, TX) and prepared as described by White (116).

3.5. IMMUNIZATIONS

Mice were immunized by the intranasal, oral or intravenous route 1-3 times at 5-day intervals, unless otherwise mentioned, following the procedures reported earlier (117), with a combination of the either R15K peptide or OVA protein at 100 ug/mouse/dose and the adjuvant CT-2* at 1ug/mouse/dose, synthetic glycolipid α -GalCer at 2 ug/mouse/dose, or NV-VLP at 10ug/mouse/dose. For intranasal immunizations, mice were anaesthetized by the intraperitoneal (ip) injection of a ketamine-xylazine mixture (10mg/mL ketamine and 1mg/mL xylazine in bacteriostatic water), and 10 uL of the adjuvant-antigen mixture diluted in 1x PBS was introduced into each nostril. For oral immunizations, mice were anaesthetized ip with the ketamine-xylazine mixture and 100 uL of the adjuvant-antigen mixture diluted in 1x PBS was introduced directly to the mouth (also referred to as sublingual immunization). For intravenous immunizations, 200 uL of the adjuvant-antigen mixture diluted in 1x PBS was injected into the tail vein of the mouse. At various time-points post immunization mice were sacrificed and cell suspensions were prepared from the spleen by homogenization while cell suspensions were prepared from the lung, liver and lymph nodes by enzymatic dissociation for one hour incubation with 1 mg/mL collagenase type IV (Sigma, St. Louis, MO) solution re-suspended in complete RPMI medium. When the lungs were collected, the mouse was perfused with 10 mL of 1x PBS prior to tissue collection. Lymphocytes from liver were further isolated by centrifugation through a percoll (Sigma, St. Louis, MO) gradient where 44% and 67% percoll solutions were made with serum free RPMI. Cells were suspended in the in the 44% percoll solution and the 67% percoll solution was layered below using a 14 gauge pipetting needle (Fisher Scientific, Waltham, MA). The solution was then centrifuged for 20 minutes at 2700 rpm, and the interphase was collected and

washed in RPMI. Red blood cells were removed by incubation with ACK lysing buffer (Lonza Bioscience, Basel, Switzerland).

3.6 ANALYSIS OF ANTIGEN-SPECIFIC CYTOLYTIC ACTIVITY BY THE ⁵¹Cr RELEASE ASSAY

The cytotoxic T lymphocyte (CTL) responses of splenocytes from immunized mice were assayed as described previously (110). Briefly, spleen cells were re-stimulated for 5 days with either the R15K or OVA peptide depending on the immunization. These effector cells were tested for cytolytic activity against ⁵¹Cr-labeled syngeneic P815 (for R15K immunization in Balb/C mice) or EL-4 (for OVA immunization in C57BL6 mice) target cells that were pre-incubated with either medium alone or stimulating peptide. The effector cells were incubated with the labeled target cells at different ratios for 5 hours at 37°C and 5% CO₂ atmosphere and then the supernatants were harvested and analyzed for radioactivity on a Wallac CliniGamma 1272 counter (Perkin Elmer, Waltham, MA). The percentage (%) of specific lysis was calculated using the following formula: % specific lysis = (experimental release - spontaneous release) / (maximum release - spontaneous release) x 100, where the spontaneous release represents the radioactivity obtained when the target cells were incubated in culture medium without effectors and maximum release represents the radioactivity obtained when the target cells were lysed with 1 % Triton X-100 (Sigma, St Louis, MO).

3.7. PREPARATION OF TARGET CELLS EXPRESSING HIV ENV gp160

The P815 target cells were infected with control vaccinia virus (vSC8) or recombinant vaccinia virus expressing the envelope gene from HIV-1IIIB (vPE16), at a multiplicity of

infection of 10 as described earlier (118). After overnight infection, the cells were washed and labeled with ^{51}Cr (Perkin Elmer, Waltham, MA) and used as targets in the CTL assay described above. The recombinant vaccinia viruses were obtained through the AIDS Research and Reference Reagent Program, division of AIDS, NIAID, NIH.

3.8. IFN γ ELISPOT ASSAY

Cells isolated from spleen, cervical lymph nodes (CLN), mesenteric lymph nodes (MLN), lung and mediastinal lymph nodes (MdLN) of immunized mice were subjected to ELISpot assay for antigen specific interferon gamma (IFN γ)-producing cells as described earlier (119) using the reagent kit from BD Biosciences (San Jose, CA). The spots, representing individual IFN γ -producing cell as spot forming cells (SFC), on the membrane were enumerated by the independent agency Zellnet Consulting Inc., New York, NY using the KS-ELISPOT automatic system (Carl Zeiss Inc., Thornwood, NY). Responses were considered positive when they were above 10 SFC/well and at least double the number obtained in cells cultured with medium alone.

3.9. FLUORESCENCE LABELED ANTIBODIES AND FLOW CYTOMETRY

Single Cell suspensions prepared from the various tissues were stained with fluorescent labeled antibodies to different cell surface markers: FITC-conjugated anti-PD-1 (clone J43, eBioscience, San Diego, CA) or FITC-conjugated anti-CD11b (clone M1/70), Pacific Blue-conjugated anti-CD3 (clone 500A2), APC-conjugated anti-CD11c (clone HL3), PE-conjugated anti-CD86 (clone GL1). All the reagents, unless otherwise mentioned, were purchased from BD Biosciences, San Jose, CA. The APC-conjugated mouse CD1d tetramer loaded with PBS57 was obtained from the NIAID tetramer facility at Emory

University, Atlanta, GA. NKT cells were stained first with Aqua Live/Dead reagent (Invitrogen, Carlsbad, CA) according to manufacture directions, and then cells were washed and incubated with the CD1d tetramer for 30 minutes in the dark at 37°C. Cells were then incubated with a combination of surface markers for an additional 30 minutes at 4°C, and then washed and fixed with BD Cytotfix/Cytoperm Buffer (BD Biosciences, San Jose, CA) for 10 minutes at 4°C. DCs were incubated with a combination of surface markers for 30 minutes at 4°C. After staining all cells were analyzed on an LSRII (BD Biosciences, San Jose, CA) and the data was processed using FlowJo software (Tree Star Inc, Ashland, OR). For NKT cell analysis, lymphocytes were first gated using the forward scatter and side scatter plots. Next live cells were gated on using side scatter and Aqua staining. Finally, the NKT cell population was determined by gating on cells positive for PB-CD3 and CD1d Tetramer and these cells were analyzed further for surface marker expression and cytokine production. For DC analysis, cells were first gated using forward scatter and side scatter, and then DCs were specifically gated on by their CD11c⁺ phenotype.

3.10. INTRACELLULAR CYTOKINE STAINING

For intracellular cytokine staining all cells were incubated with GolgiPlug (BD Biosciences, San Jose, CA) in complete medium for 4.5 hours before any cellular staining. Cells were stained for surface markers and fixed as described above. Cells were then washed and incubated with cytokine antibodies in 1x BD Perm/Wash Buffer (BD Biosciences, San Jose, CA) for 60 minutes at 4°C. The cytokine specific antibody used in these experiments was PE-conjugated anti-IFN γ (clone XMG1.2, BD Biosciences, San Jose, CA). Cells were then washed two more times in the Perm/Wash

buffer and fixed in BD Cytofix/Cytoperm buffer, and samples were analyzed on the LSRII as described above.

3.11. OT-1 PROLIFERATION ASSAY

Splenocytes were collected from OVA transgenic mice (OT-1) and cultured for 5 to 8 days in RPMI complete medium supplemented with 10% FBS, and 30 U/mL hIL-2 (Chiron, Emeryville, CA), cells were initially cultured with 1 ug/mL OVA peptide, but no additional peptide was added in subsequent sub-culturing of the cells. These cells were fixed in 0.75% paraformaldehyde (Sigma, St Louis, MO) and were co-cultured at 1:10 ratio with cells from immunized mice for 48 hrs at 37C. To each culture, 1 uCi of ³H-Thymidine (Perkin Elmer, Waltham, MA) was added for the final 18hrs and cells were harvested and measured for ³H incorporation.

3.12. EX VIVO PRESENTATION OF α -GALCER

Cells isolated from immunized mice were used as presenters of α -GalCer for co-culturing with the NKT cell hybridoma DN32.D2 for 24 hours at a 10:1 ratio.

Alternatively, cells from different tissues of immunized mice were purified using the MACs beads for CD11c+ or B220+ cells as described by Thapa (120). Briefly, cells were stained with either anti-CD11c-PE or anti-B220-PE and then incubated with anti-PE magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were positively selected on an autoMACs separator (Miltenyi Biotec, Bergisch Gladbach, Germany). Aliquots (5×10^5) of these cells were co-cultured with 1×10^5 NKT hybridoma cells. The NKT cell activation was determined in terms of the release of IL-2 which was measured using the CTLL assay as described in the literature (121). Briefly,

supernatants were collected from the co-cultures described above, serially diluted, and incubated with 5×10^3 CTLL cells for approximately 40 hours at 37°C. To each sample, 1 uCi of ^3H -Thymidine was added for the final 16hrs and cells were harvested and measured for ^3H incorporation.

3.13. STATISTICAL ANALYSIS

The data were analyzed for statistical significance using the student's t-test for paired samples and a P-value of <0.05 was considered significant. Data shown is representative of results obtained from individual experiments. Experiments were repeated three times with similar results obtained.

CHAPTER 4

RESULTS

4.1 EFFECTIVENESS OF α -GALCER AS AN ADJUVANT AFTER MUCOSAL DELIVERY:

The role of α -GalCer as a mucosal adjuvant was assessed by determining its ability to prime mucosal and systemic immune responses to co-administered antigens/immunogens. In addition, the dosing route and schedule as well as the effects of primary and booster immunization using α -GalCer as adjuvant were investigated.

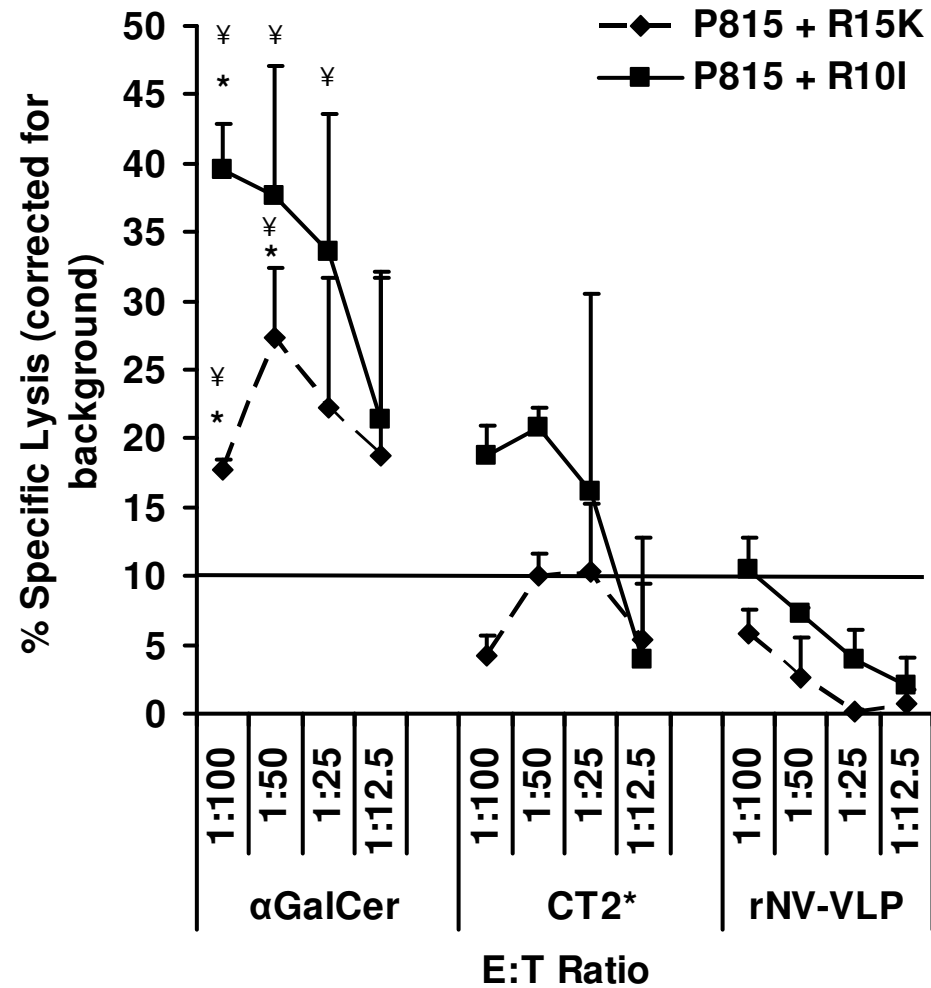
4.1.1 Comparison of different promising mucosal adjuvants.

First, the effectiveness of α -GalCer as a mucosal adjuvant to prime antigen-specific immune responses was tested in comparison to a mutant cholera toxin (CT2*) that has been previously studied in this lab (35) and also a virus like particle (VLP) formulation of the gastrointestinal virus Norwalk virus (NV-VLP). The NV-VLP was originally developed as a potential vaccine candidate to induce NV specific immune responses and protective immunity against NV (122), although the VLP was administered without any additional adjuvants, suggesting that NV-VLP may exhibit adjuvant potential. Each of these reagents was administered by the intranasal route after mixing with a model immunogen, R15K (RIQRGPGRAFVTIGK), a CTL-inducing HIV-1 envelope peptide (110, 117). Separate groups of Balb/C mice were immunized with 2 doses of the R15K peptide admixed with each of these reagents by the intranasal route at 5 day intervals. The mice were sacrificed and splenocytes were harvested 5 days after the 2nd immunization. Splenocytes were re-stimulated in vitro for 5 days with the immunizing peptide and

assessed for CTL activity by the standard chromium release assay. Among the three adjuvant candidates tested, α -GalCer was found to induce significantly higher antigen-specific CTL immune responses ($p < 0.05$, based on student's t-test analysis) when compared to the other two adjuvants tested (Fig. 4.1.1). Based on these results it is concluded that among the three adjuvant candidates tested, α -GalCer was superior and a detailed analyses of the adjuvant potential of α -GalCer is then conducted starting with determining the number of doses of adjuvant-antigen mixture that would be necessary to induce broad systemic and mucosal immune responses to the co-administered antigen.

Fig. 4.1.1: Effectiveness of different adjuvant candidates delivered by the intranasal route for inducing immune responses to co-administered peptide antigen. Balb/C mice were immunized with 100ug of the model HIV peptide, R15K, admixed with either CT2* (1ug), NV-VLP (10ug) or α -GalCer (2ug) as the adjuvant by the intranasal route twice at 5-day interval, as shown in the immunization scheme (A). The mice were sacrificed 5 days after the booster dose and splenocytes were harvested and re-stimulated with the immunizing peptide for 5 days. The antigen-specific cytolytic activity of the splenocytes was assessed by the chromium release assay (B), where syngenic P815 target cells were pulsed in vitro with chromium and either medium alone, the immunizing peptide (R15K, diamond) or the specific CTL epitope within the immunizing peptide (R10I, square). These target cells were then co-cultured with the re-stimulated effector cells from immunized mice at different effector to target (E:T) ratios for 5 hours, and the specific lysis was determined as described in the Methods section. The % specific lysis values shown are adjusted to un-pulsed P815 target cells. The horizontal line indicates the cut-off value for positive antigen-specific lysis (10%). Significance was determined with the student's t-test where the * represents significance ($p < 0.05$) between groups of mice immunized with R15K admixed with α -GalCer compared to mice immunized with R15K admixed with CT2* and ¥ represents significance ($p < 0.05$) between groups of mice immunized with R15K admixed with α -GalCer compared to mice immunized with R15K admixed with NV-VLP. This experiment was performed with 2 mice per groups and repeated once.

Fig. 4.1.1: Effectiveness of different adjuvant candidates delivered by the intranasal route for inducing immune responses to co-administered peptide antigen.



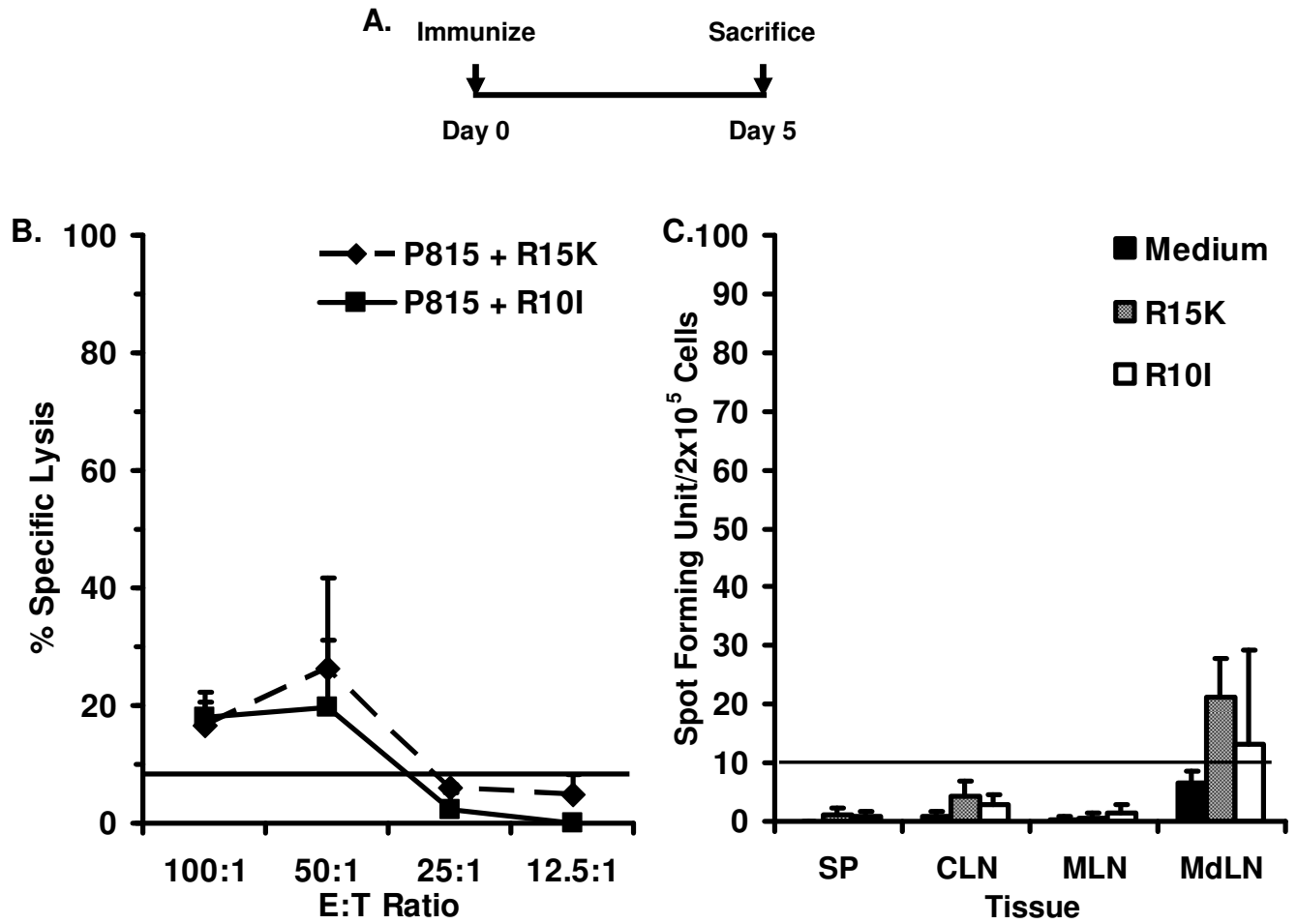
4.1.2 Intranasal Immunization with a primary dose of α -GalCer and the HIV model peptide elicits antigen-specific immune responses.

The effectiveness of α -GalCer as an adjuvant was analyzed to determine first whether a primary dose delivered by the intranasal route would be sufficient for inducing antigen-specific immune responses at different systemic and mucosal tissues. For this, α -GalCer was admixed with the model CTL-inducing HIV peptide R15K and delivered by the intranasal route to groups of Balb/C mice (n=3). The mice were sacrificed 5 days after the primary immunization, and the lymphocytes isolated from the spleen and different lymph nodes were assessed for CTL activity and/or the numbers of IFN γ -producing cells specific to the immunizing peptide R15K as well as a shorter 10 amino acid peptide R10I (RGPGRAFVTI) representing the specific CTL epitope located within the immunizing peptide (Fig. 4.1.2). A moderate induction of CTL activity was observed in the spleen that was specific to R15K and also R10I (Fig. 4.1.2B). However, only low levels of peptide-specific interferon gamma (IFN γ) producing cells were observed in the lung draining mediastinal lymph nodes (MdLN) but not in the spleen, cervical draining lymph nodes (CLN) or gut associated lymph mesenteric lymph nodes (MLN) (Fig. 4.1.2C).

Fig. 4.1.2: Induction of antigen-specific cellular immune responses after primary intranasal immunization with the α -GalCer adjuvant admixed with HIV peptide.

Balb/C mice were immunized with a mixture of α -GalCer (2ug) and the HIV-1 envelope peptide R15K (100ug) and five days later the mice were sacrificed to collect lymph nodes and spleen tissues as shown in the immunization scheme (A). Single cell preparations from spleens were analyzed for antigen-specific CTL activity by the standard chromium-release assay employing the syngeneic P815 target cells pulsed in vitro with either the immunizing peptide (R15K) or the CTL epitope peptide (R10I) at different effector to target cell (E:T) ratios (B). The % specific lysis values shown are adjusted to un-pulsed P815 cells. The horizontal line indicates the cut-off value for positive antigen-specific lysis (10%). Cells harvested from spleen and MLN were also analyzed for the numbers of IFN γ producing cells using the standard cytokine ELISpot assay (C) by incubating for 48 hours with media alone (Black), the immunizing peptide R15K (Grey), or the specific CTL epitope R10I (White). The numbers of IFN γ producing cells were enumerated from the total 2×10^5 input cells. The horizontal line indicates the cut-off value for positivity of antigen-specific IFN γ -producing cells (10). This experiment was performed with 3 mice per group and repeated 3 times.

Fig. 4.1.2: Induction of antigen-specific cellular immune responses after primary intranasal immunization with the α -GalCer adjuvant admixed with HIV peptide.



4.1.3 Intranasal booster immunization with α -GalCer and the HIV peptide elicits strong peptide-specific systemic and mucosal immune responses.

Since only weak immunogenicity was observed after a primary dose of the HIV model peptide and α -GalCer delivered by the intranasal route, it was necessary to determine whether booster doses of the antigen-adjuvant mixtures would increase the immunogenicity of the α -GalCer adjuvant. In these studies, one or more doses of the adjuvant-antigen mixture delivered subsequent to the initial/primary immunization are referred to as booster doses or booster immunizations. Mice were immunized by the intranasal route with the α -GalCer + R15K mixture with a primary dose followed by a booster dose 5 days later (Fig. 4.1.3A). Mice were sacrificed 5 days after the booster dose and cells harvested from the spleen and lymph nodes were analyzed for antigen-specific cellular immune responses. This immunization regimen resulted in strong induction of antigen-specific CTL activity in the spleen (Fig. 4.1.3B). Additionally, we observed antigen-specific IFN γ producing cells within the mucosal tissues such as the MLN and CLN as shown in Fig. 4.1.3C. These data demonstrate that an intranasal prime-boost immunization regimen with α -GalCer ad-mixed with the R15K peptide was effective in priming not only a strong systemic immune response, but also a broader mucosal immune response in multiple tissues, including the more distal mucosal site, the MLN. Furthermore, these results suggest that intranasal booster immunization with α -GalCer and the HIV peptide is necessary to induce broader antigen-specific mucosal immunity.

Based on the observed improvements in the antigen-specific immune responses resulting from a single booster immunization, the effect of a 2nd booster dose of

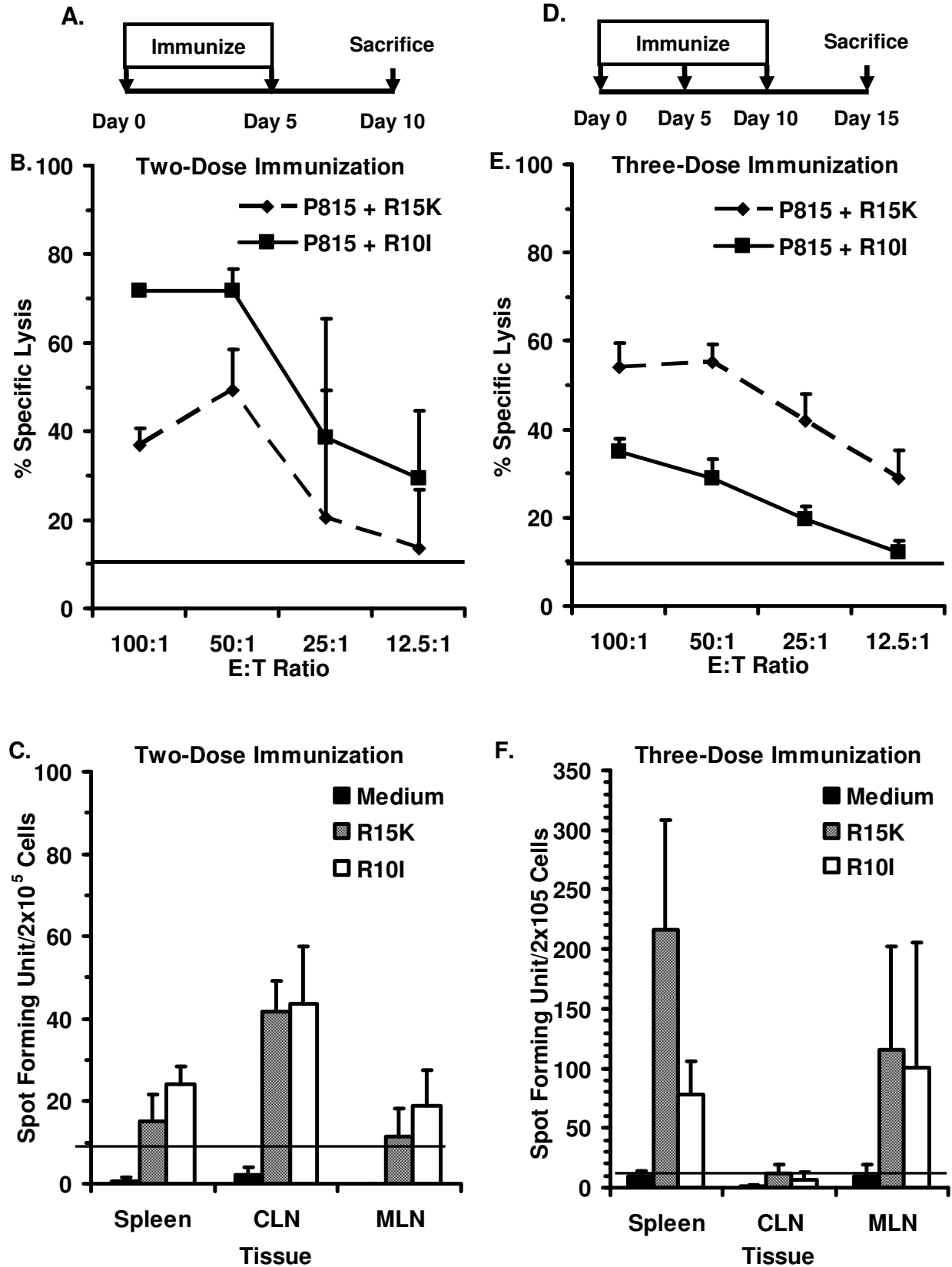
adjuvant-antigen mixture delivered by the intranasal route was tested to determine whether it would further enhance the immune responses. Mice were immunized with α -GalCer and R15K mixture three times at 5-day interval and the cells collected from spleen and the various mucosal tissues were assessed for antigen-specific immune responses (Fig. 4.1.3D). As shown in Fig. 4.1.3E, strong antigen-specific CTL response, at comparable levels to that seen after the 2-dose immunization scheme were observed in the spleens. Similarly, peptide-specific IFN γ producing cells were observed in the spleen and the MLN (Fig. 4.1.3F). These results suggest that booster immunization with the antigen and α -GalCer mixture by the intranasal route is necessary for efficient induction of strong antigen-specific systemic as well as cellular immune responses with no significant improvement or hindrance with additional doses.

Fig. 4.1.3: Enhanced induction of cellular immune responses after booster

intranasal immunization with α -GalCer and HIV peptide. Groups of Balb/C mice were immunized with either two doses (A) or three doses (D) of the mixture of α -GalCer (2ug) and R15K peptide (100ug) and cells were collected from the spleen and mucosal tissues. Cytolytic activity was assayed in the spleen cells using syngeneic P815 target cells pulsed in vitro with the immunizing R15K peptide and the CTL epitope peptide R10I (B and E) and adjusted by subtracting the values for un-pulsed target cells. The horizontal line indicates the cut-off value for positive antigen-specific lysis (10%). The IFN γ producing cells were enumerated within the spleen and MLN by cytokine ELISpot assay (C and F). The horizontal line indicates the cut-off value for positivity of antigen-specific IFN γ -producing cells (10). This experiment was performed with 3 mice per group and repeated 3 times.

Fig. 4.1.3: Enhanced induction of cellular immune responses after booster

intranasal immunization with α -GalCer and HIV peptide.

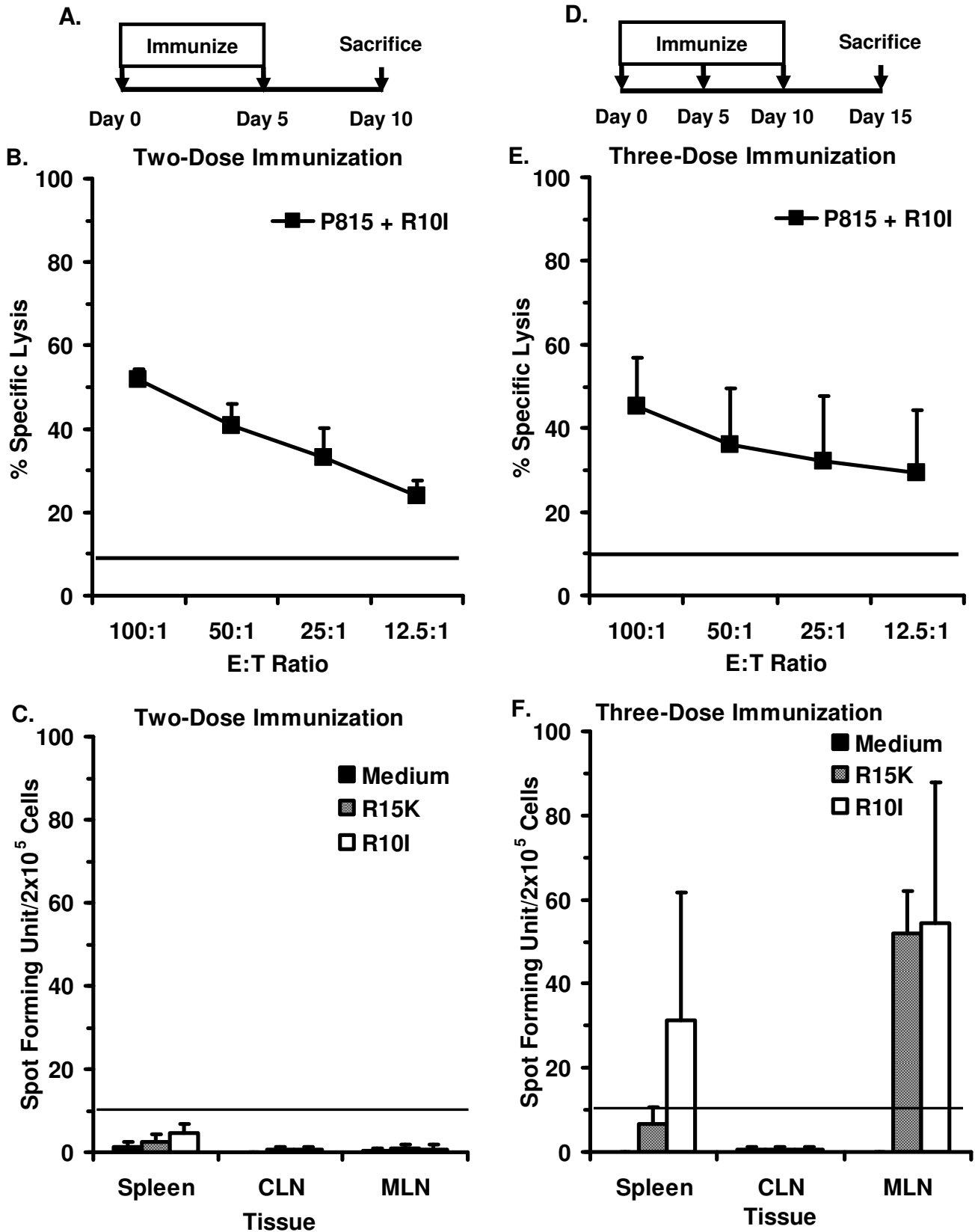


4.1.4 Primary and booster immunizations with α -GalCer and the model HIV peptide administered by the oral route results in strong peptide-specific systemic and mucosal immune responses.

Since oral immunization is a more practical strategy for vaccine delivery, we tested whether α -GalCer will be an effective adjuvant delivered by this route. For this, the oral administration of the α -GalCer adjuvant along with the HIV peptide was assessed for the induction of antigen-specific cellular immune responses by delivering the mixture directly into the mouths of Balb/c mice using a pipette (under the tongue or sub-lingual). After the immunization delivered twice at 5 days apart (Fig. 4.1.4A), strong antigen-specific CTL responses were observed in the spleens of the mice (Fig. 4.1.4B). However, antigen-specific IFN γ producing cells were not detected in any of the tissues tested (Fig. 4.1.4C). These results were similar to those observed above in mice after a single intranasal immunization. Therefore, we next tested an oral immunization regimen consisting of a primary dose of α -GalCer and R15K in addition to 2 booster doses administered at 5-day intervals (Fig. 4.1.4C). In this regimen also strong CTL responses were observed in the spleen (Fig. 4.1.4D). Additionally, significant enhancement of antigen-specific IFN γ producing cells was observed in the spleen and MLN (Fig. 4.1.4E) but, not in the CLN. These results are consistent with the oral route of immunization, in which the MLN is a more relevant draining lymph node relative to the CLN. Therefore, these data suggest that a primary dose immunization along with 2 booster immunizations is necessary for the induction of systemic and mucosal antigen-specific immune responses after oral delivery of α -GalCer and R15K.

Fig. 4.1.4: Enhanced induction of cellular immune responses after repeated oral immunization with α -GalCer and HIV peptide. Groups of Balb/C mice were immunized by the oral route with either two doses (A) or three doses (D), of a mixture of α -GalCer (2ug) and R15K (100ug) and cells collected from the spleen were analyzed for cytolytic activity (B and E) against syngenic P815 target cells pulsed with the CTL epitope peptide R10I; The horizontal line indicates the cut-off value for positive antigen-specific lysis (10%). Antigen-specific IFN γ producing cells were enumerated in the spleen and cervical as well as mesenteric lymph nodes (CLN and MLN, respectively) by the cytokine ELISpot assay (C and F). The horizontal line indicates the cut-off value for positivity of antigen-specific IFN γ -producing cells (10). Data shown are average values from three separate experiments corrected for background values with un-pulsed target cells for the cytolytic assay. This experiment was performed with 3 mice per group and repeated once.

Fig. 4.1.4: Enhanced induction of cellular immune responses after repeated oral immunization with α -GalCer and HIV peptide.

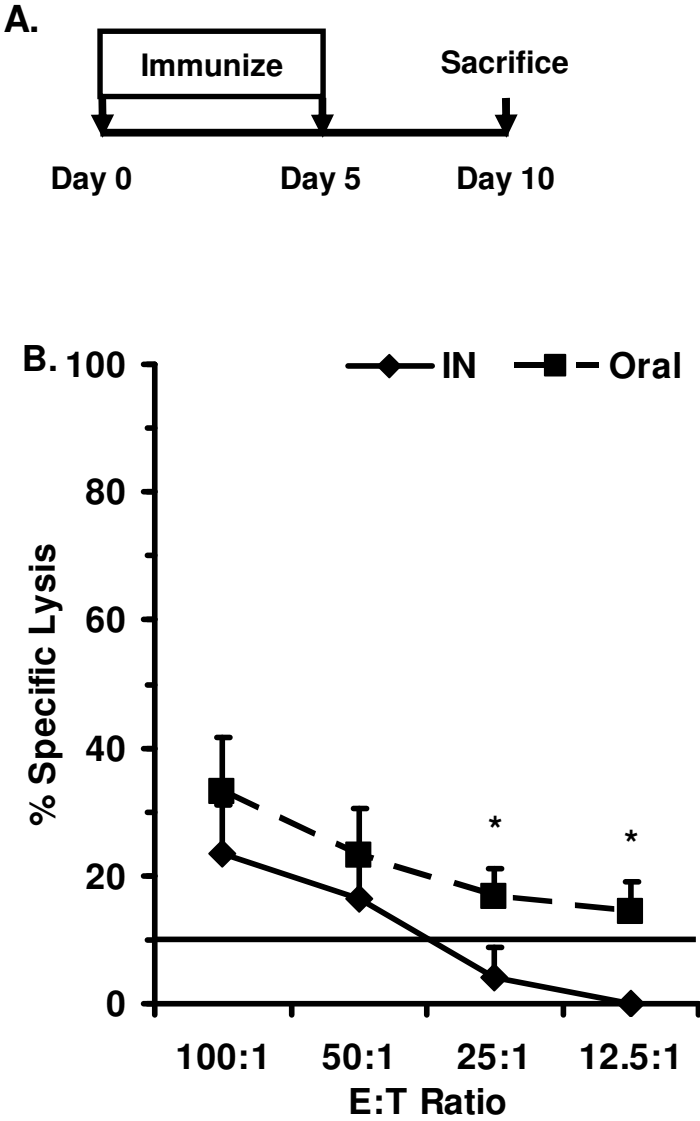


4.1.5 Immunization with the model HIV peptide and α -GalCer by either the oral or intranasal route leads to immune recognition of the cognate HIV envelope protein.

Next, the ability of α -GalCer to prime antigen-specific immune responses that can recognize virally infected target cells was assessed for both oral and intranasal routes of immunization. Splenocytes isolated from mice immunized with the antigen-adjuvant mixture showed strong CTL activity against target cells infected with a recombinant vaccinia virus expressing the HIV-1 envelope protein gp160 (VPE16), when compared to cells infected with the control vaccinia virus vSC8 (Fig. 4.1.5B). Mice immunized by either of the two mucosal routes tested were able to elicit envelope specific lysis of target cells with no significant difference between the two immunization routes. These results demonstrate the effectiveness of the mucosal immunization employing α -GalCer as an adjuvant for R15K peptide to prime cognate antigen-specific CTL responses.

Fig. 4.1.5: Induction of cognate protein-specific cellular immune responses after oral or intranasal immunization with α -GalCer and HIV antigen. Groups of Balb/C mice were immunized with two doses of α -GalCer (2ug) and the HIV envelope peptide R15K (100ug) by either the oral or intranasal (IN) route (scheme as shown in A). Spleen cells were re-stimulated for 5 days with the immunizing peptide and the cytolytic activity determined using the syngenic P815 target cells infected overnight with either a control vaccinia virus (vSC8) or a recombinant vaccinia virus expressing the HIV envelope protein gp160 (vPE16) employing different effector to target cell ratios as shown (B). The horizontal line indicates the cut-off value for positive antigen-specific lysis (10%). Significance was determined with the student T-test where the * represents significance ($p < 0.05$) between groups of mice immunized by the intranasal versus the oral route. Data shown is corrected for background values with the vSC8 infected target cells. This experiment was performed with 3 mice per group and repeated 3 times.

Fig. 4.1.5: Induction of cognate protein-specific cellular immune responses after oral or intranasal immunization with α -GalCer and HIV antigen.



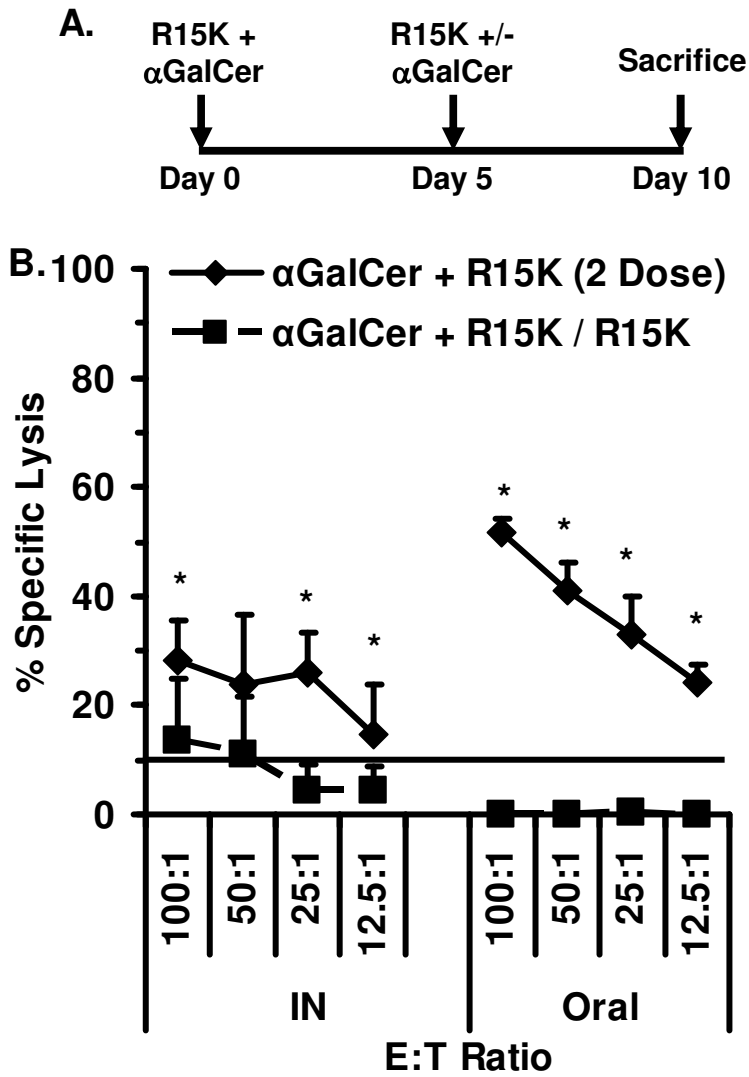
4.1.6 Booster Doses of α -GalCer delivered via mucosal routes are necessary for priming antigen-specific CTL responses.

Thus far, results obtained as described above suggest that repeated dosing of the α -GalCer adjuvant admixed with antigen delivered by intranasal or oral mucosal route induces strong antigen-specific immune responses without undue adverse effects on the cellular immune responses. However, we wanted to determine whether α -GalCer was necessary in the booster dose, by comparing the antigen-specific immune responses of a group of mice both primed and boosted with α -GalCer admixed with peptide to a group primed with α -GalCer plus peptide and boosted with peptide alone (Fig. 4.1.6A). As shown in Fig. 4.1.6B at most of the E:T ratios tested, cells from mice that received the booster dose containing α -GalCer showed significantly higher levels of antigen-specific lysis than those from mice that received a booster dose of the peptide alone. Therefore, after intranasal as well as oral routes of immunization, we observed that the second dose of α -GalCer is in fact necessary for the induction of peptide-specific CTL responses.

Fig. 4.1.6: Activation of cellular immune responses after mucosally delivered booster immunization of HIV antigen with or without the α -GalCer adjuvant.

Groups of Balb/C mice were administered a primary immunization by the oral or intranasal route with a mixture of α -GalCer (2ug) and R15K (100ug) followed by a booster immunization with either the R15K peptide (100ug) alone (α -GalCer +R15K/R15K) or along with α -GalCer (2ug) on day 5 before sacrificing on day 10 [α -GalCer + R15K (2 Dose), scheme as shown in A]. Spleen cells collected from mice immunized by the oral or intranasal route were re-stimulated for 5 days with the peptide and analyzed for cytolytic activity using syngenic P815 target cells pulsed in vitro with the R15K peptide (B). The horizontal line indicates the cut-off value for positive antigen-specific lysis (10%). Significance was determined with the student T-test where the * represents significance ($p < 0.05$) between groups of mice immunized with R15K peptide alone versus groups of mice immunized with R15K along with α -GalCer. Data shown are average values after subtracting background values from un-pulsed target cells. This experiment was performed with 3 mice per group and repeated once.

Fig. 4.1.6: Activation of cellular immune responses after mucosally delivered booster immunization of HIV antigen with or without the α -GalCer adjuvant.



4.2 THE MECHANISM OF MUCOSAL ADJUVANTICITY OF α -GALCER:

Results obtained thus far show the effectiveness of α -GalCer to serve as a strong mucosal adjuvant for the priming as well as boosting of systemic and mucosal immune responses specific to co-administered antigens delivered by the intranasal or oral routes (96). We sought to understand the underlying mechanism by analyzing the kinetics and functional properties of NKT cells, relative to both priming and boosting by the intranasal route utilizing the model antigen chicken ovalbumin (OVA).

4.2.1. Intranasal immunization using α -GalCer adjuvant primes T cell responses to co-administered antigen by inducing activation of NKT cells and DC.

Groups of mice (n=3) immunized with either OVA and α -GalCer (α -GalCer group) or OVA alone (control group) were sacrificed on days 1, 3 and 5 post-immunization (Fig. 4.2.1A), and the cells isolated from spleen, liver, lung and lung-draining mediastinal lymph nodes (MdLN) were analyzed for phenotypic and functional characteristics of NKT cells and dendritic cells (DC). By day 1 post-immunization, relative to the control group (OVA), we observed higher percentages of IFN γ -producing NKT cells in multiple tissues in the α -GalCer group, with significant increase in the lung and liver tissues (Figs. 4.2.1B and 4.2.1C). We also observed expansion of NKT cells in the α -GalCer group of mice with the peak levels at day 3 post-immunization in the spleen, while that in the lungs continued to increase till day 5 post-immunization (Figs. 4.2.1D and E). Thus, intranasal immunization employing α -GalCer as an adjuvant induced activation and expansion of NKT cells in multiple tissues with the highest and most prolonged responses observed in the lungs. It has been reported that α -GalCer administered by the systemic route (e.g. intravenous or intraperitoneal) potently stimulates NKT cells and leads to activation of

DC that in turn induce efficient presentation of the co-administered antigen (54, 123). Consistent with this notion, higher numbers of CD11c⁺ DC expressing the CD86 activation marker were observed within the spleen, lung and MdLN on day 1 after intranasal immunization in the α -GalCer group when compared to that in the control OVA group (Figs. 4.2.2A and B). Importantly, the increase in DC activation observed in the lung draining MdLN, was accompanied with increased antigen presentation function, determined in terms of stimulation of the growth of co-cultured OVA-transgenic OT-1 mouse splenocytes (Fig. 4.2.2C).

Fig. 4.2.1: Activation and expansion of NKT cells after single intranasal

administration of α -GalCer. Mice were immunized by the intranasal route with either OVA alone or admixed with α -GalCer (α -GC + OVA) and sacrificed 1, 3 and 5 days post immunization (A). Cells isolated from the various tissues as shown were stained with fluorescence labeled reagents: anti-CD3-PB, NKT tetramer-APC, anti-IFN γ -PE and Aqua live/dead stain. The live NKT+ CD3+ cells producing IFN γ were enumerated for the four tissues on day 1 post-immunization (B). Average values along with standard deviation for all the mice in the two different groups tested are shown to depict the changes in the percentages of IFN γ producing NKT cells from day 1 to day 5 after intranasal immunization (C). Representative data from one mouse shows the percentage of NKT tetramer+ cells measured from total CD3+ cells to determine the amount of NKT cell expansion at days 1, 3 and 5 post-immunization (D). The average values of NKT tetramer + cells out of total CD3+ cells were determined along with standard deviation to show the expansion of NKT cells in all the mice tested between days 1 to 5 after intranasal immunization (E). Significance was determined with the student T-test where the * represents significance ($p < 0.05$) between OVA alone and α -GalCer + OVA. This experiment was performed with 3 mice per group and repeated 3 times.

Fig. 4.2.1: Activation and expansion of NKT cells after single intranasal administration of α -GalCer

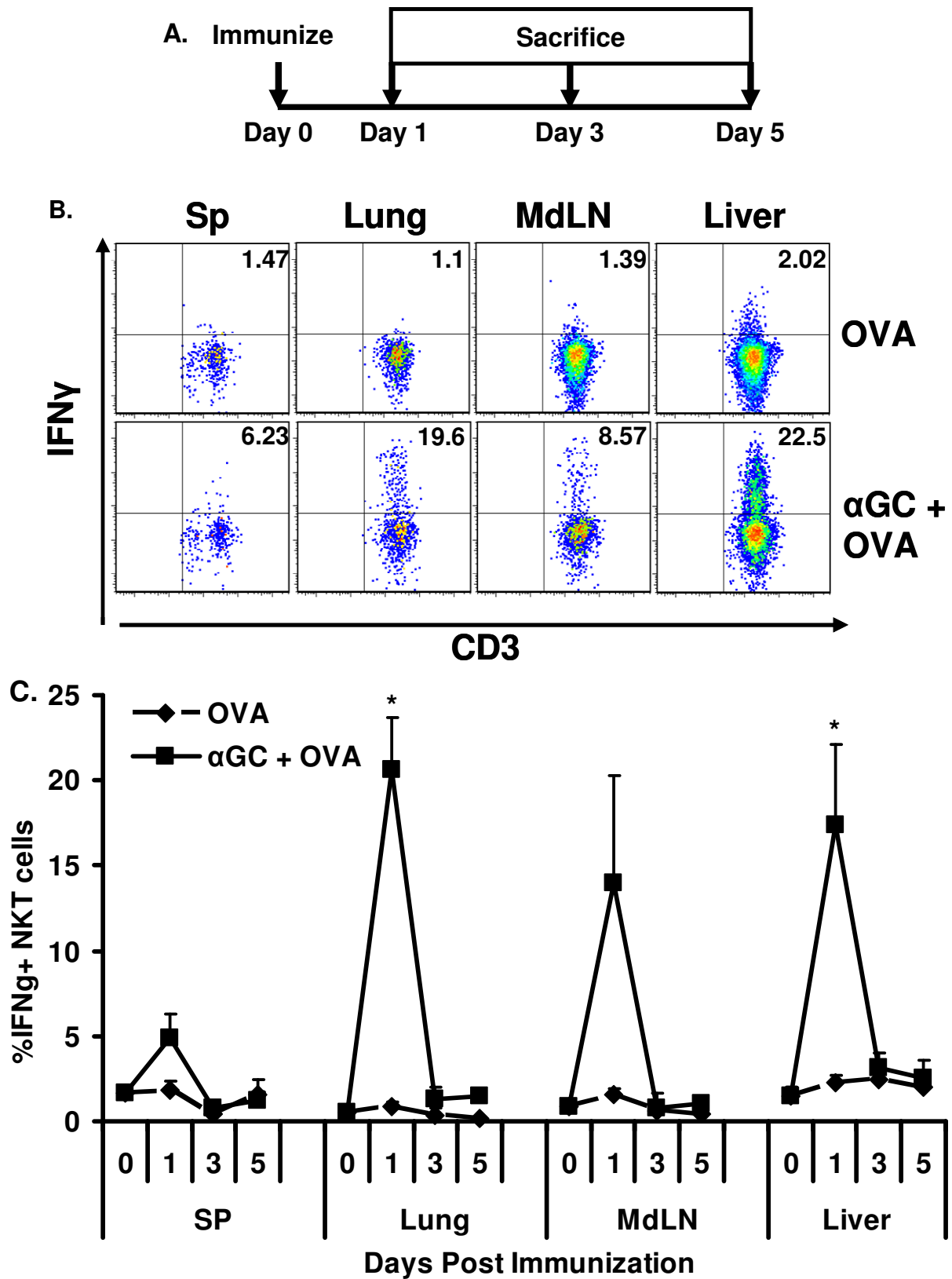


Fig. 4.2.1: Activation and expansion of NKT cells after single intranasal administration of α -GalCer Cont.

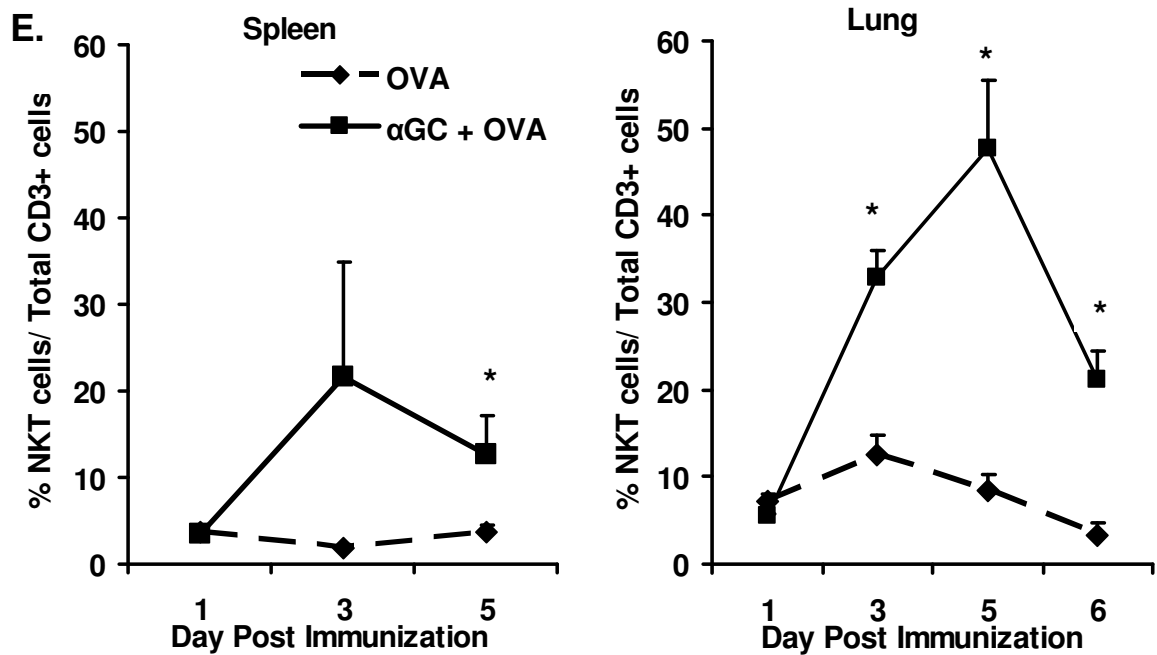
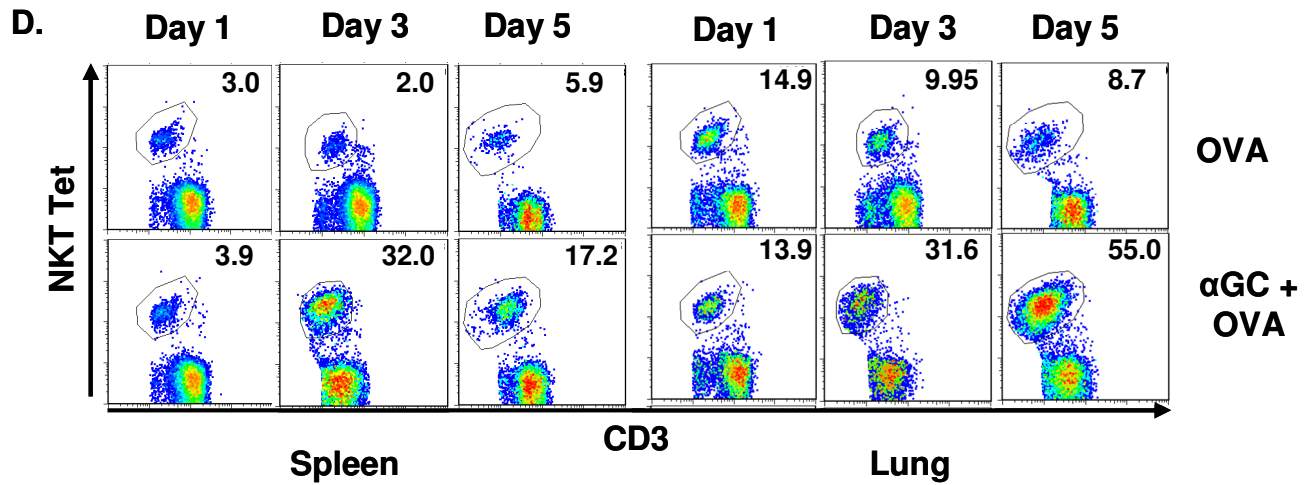
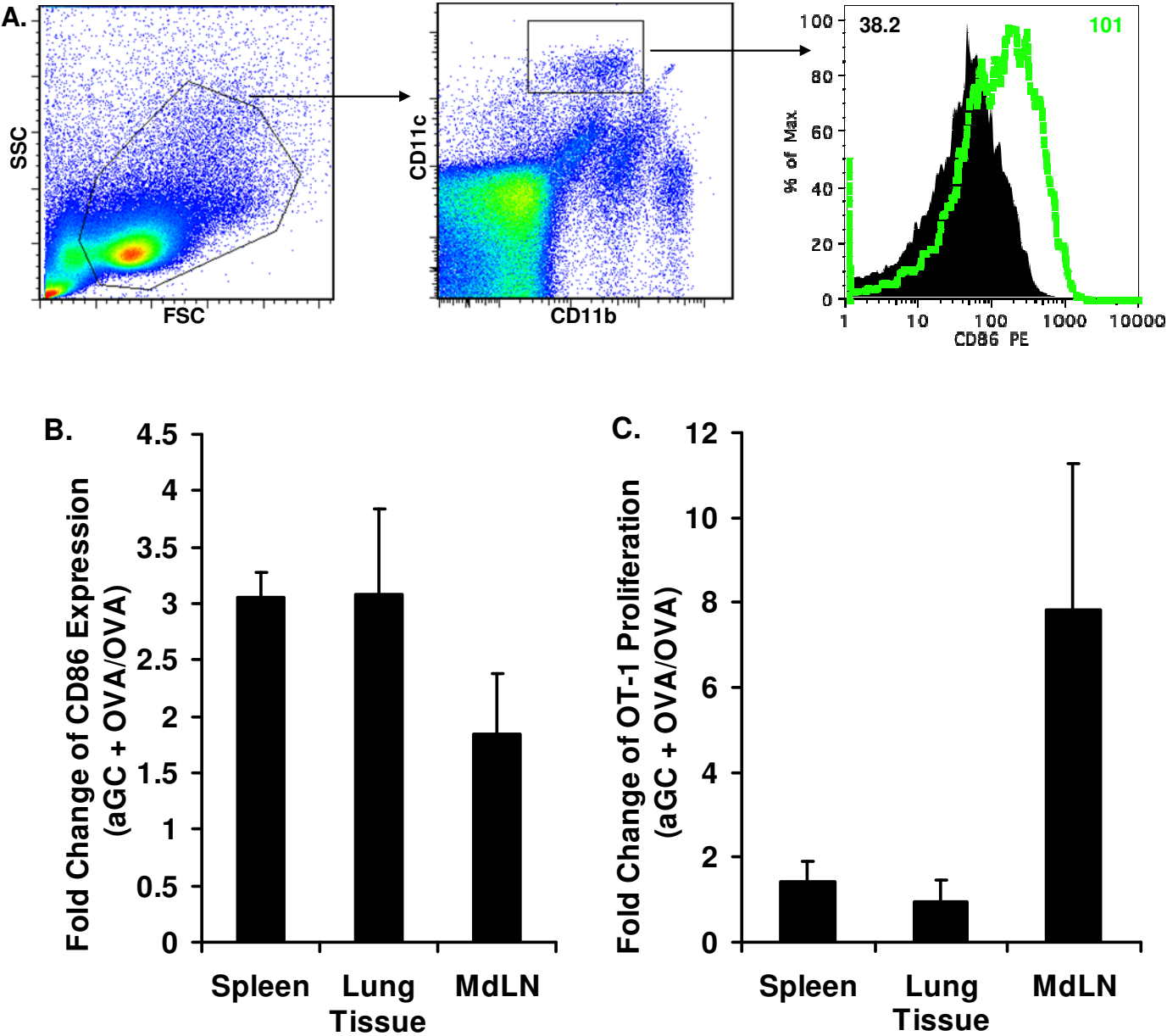


Fig. 4.2.2: Activation of DC after a single intranasal administration of α -GalCer.

Mice were immunized by the intranasal route with either OVA alone or admixed with α -GalCer (α -GC + OVA) and sacrificed 1 day post immunization. Cells isolated from the spleen, lung and MdLN were stained with fluorescent labeled antibodies: anti-CD11b-FITC, anti-CD11c-APC and anti-CD86-PE. The CD11c⁺ cells were evaluated for activation in terms of the level of CD86 expression. Panel A shows the gating tree for the CD11c⁺ DC expressing the activation marker CD86 and a representative histogram for percentage of activated CD11c⁺ cells in the spleen in one mouse each, immunized with ova alone (filled histogram) or α -GC + OVA (open histogram). Panel B is the average data with standard deviation values for the fold change of CD86 expression in the CD11c⁺ cells in the spleen, lung and MdLN of mice immunized with α -GC + OVA over the values from mice immunized with OVA alone. Additionally, an OT-1 assay was performed where cells isolated from the immunized mice were co-cultured with OVA-transgenic OT-1 splenocytes. Panel C shows data for the proliferation of the OVA-transgenic OT-1 mouse splenocytes in response to stimulation with cells isolated from the spleen, lung and MdLN of immunized mice as measured by ³H-thymidine incorporation assay. The data shown are the fold change of proliferation in terms of radio activity (cpm values) in the cells harvested from the different tissues of mice immunized with α -GC + OVA over those of mice immunized with OVA alone. This experiment was performed with 3 mice per group and repeated 3 times.

Fig. 4.2.2: Activation of DC after a single intranasal administration of α -GalCer



4.2.2. Intranasal booster immunization employing α -GalCer adjuvant increases NKT cell activation and expansion in the lung.

In order to induce potent and durable antigen-specific immunity, immunization schemes deliver one or more booster doses of the vaccine formulations at specified intervals. As shown above (Fig. 4.1.3), intranasal delivery of multiple doses of antigen admixed with α -GalCer is effective in boosting antigen-specific systemic and mucosal cellular immune responses (96). Therefore, we investigated whether booster immunization employing α -GalCer would induce enhancement and/or reactivation of NKT cells and DC. As shown in Fig. 4.2.3A, intranasal booster immunization (2nd dose of α -GalCer + OVA) was delivered on day 5 after the primary immunization, and mice were sacrificed on days 6, 8, and 10 (days 1, 3 and 5 relative to the booster dose). Single cell suspensions prepared from spleen, lung, and MdLN tissues were analyzed for phenotypic and functional properties of NKT cells. On day 6 (i.e. day 1 after the booster immunization), IFN γ producing NKT cells in the α -GalCer group significantly increased in the spleen and the lung (Fig. 4.2.3B) and between days 6 and 10 (i.e. days 1 and 5 after the booster immunization) the NKT cell population in the lung expanded significantly (Fig. 4.2.3C) when compared to those in the mice that did not receive the booster immunization or the control group of mice that received both priming and booster immunizations with OVA only. Furthermore, an increase in CD86 expression on CD11c⁺ DC was measured in the spleen, lung and MdLN after the booster dose of α -GalCer + OVA when compared to the OVA control group on Day 1 post-boost (Fig. 4.2.3D), a trend similar to that observed on day 1 after the primary immunization (Fig. 4.2.2B). Thus, the booster immunization using the α -GalCer adjuvant yielded a second wave of NKT cell expansion with IFN γ producing potential along with increase in activated DC in multiple tissues, most notably in the lung. Finally, the antigen-specific T-cell response after the booster dose was

measured on Day 10 by chromium release assay. Although the specific activity was not as high as what was observed after the HIV peptide immunizations (96), a similar pattern emerged with a primary dose of α -GalCer + OVA inducing very low levels of OVA-specific CTL activity while a booster dose of α -GalCer + OVA induced a statistically significant increase in antigen specific CTL activity in the spleen (Fig. 4.2.3E).

Since the primary immunization with α -GalCer and OVA resulted in the expansion of NKT cells that peaked at day 5 in the lung and did not decrease to base-line levels even at day 10 post immunization (Fig. 4.2.1E), the last time point examined, the second increase of NKT cells observed after the booster immunization delivered on day 5 may be a consequence of the continued effect of the priming dose of α -GalCer and not the effect of booster dose. To better understand the potential effect of the booster dose of α -GalCer to induce further activation and expansion of NKT cells, the booster immunization was delayed till day 23 post-priming and the NKT cells and DC were characterized in different tissues on days 24, 26, and 28 (i.e. days 1, 3 and 5, relative to the booster dose, Fig. 4.2.3F). Significantly higher percentages of IFN γ -producing NKT cells were observed in the spleen and lung, with a similar trend in the MdLN, at day 24 (i.e. day 1 after the booster immunization, Fig. 4.2.3G) and a further significant expansion of NKT cells was observed in the lung between days 1 and 5 after the booster immunization (Fig. 4.2.3H) when compared to either the OVA only control group or the mice that received only the priming dose α -GalCer and OVA. Additionally, CD11c⁺ DC expressed increased levels of the CD86 activation marker on day 24 (day 1 after the α -GalCer + OVA booster dose) when compared to the DC from mice from the OVA control group (Fig.4.2.3I). Finally, the antigen-specific T cell responses were assessed on day 28 (i.e. day 5 after the booster dose) and increased OVA-specific IFN γ producing cells as assessed by IFN γ ELISpot assay (Fig. 4.2.3J) were observed in the α -GalCer group

compared to the OVA alone group . These results from mice that received the priming and boosting doses of α -GalCer + OVA by the intranasal route 23 days apart (the longer immunization scheme) were similar to those observed when the priming and booster doses were delivered 5 days apart (the shorter immunization scheme). Thus, regardless of the timing of the booster dose, α -GalCer administration by the intranasal route led to repeated activation of NKT cells primarily in the lung, and to a lesser extent in the other tissues.

Fig. 4.2.3: Activation and expansion of NKT cells and DC after intranasal

administration of a booster dose of α -GalCer. Mice were immunized by the intranasal route with primary and/or booster doses of either OVA alone or admixed with α -GalCer. The booster dose was administered at day 5 after the primary immunization and the mice were sacrificed 6, 8, and 10 days post-immunization which is 1, 3 and 5 days after the booster immunization (A). Cells isolated from the various tissues as shown were stained with fluorescence labeled reagents: anti-CD3-PB, NKT tetramer-APC, anti-IFN γ -PE and Aqua live/dead stain. The live NKT $^{+}$ CD3 $^{+}$ cells producing IFN γ were enumerated for the spleen, lung and MdLN tissues of mice receiving OVA alone (OVA), 1 or 2 doses of OVA admixed with α -GalCer (α -GC + OVA 1D and α -GC + OVA 2D, respectively) on day 6 post-immunization or day 1 relative to booster immunization (B). The percentage of NKT tetramer $^{+}$ cells (NKT cells) from total CD3 $^{+}$ cells in the lung was measured to determine the amount of NKT cell expansion at days 6, 8, and 10 post-immunization (C). Activation status of DC was assessed in terms of surface CD86 expression on day 6 post-immunization and the average data is shown with standard deviation values for the fold change of CD86 expression in the CD11c $^{+}$ cells in the spleen, lung and MdLN of mice immunize with α -GC + OVA over OVA alone (D). Antigen-specific cellular immune responses were assessed for cytolytic activity by the standard chromium release assay using syngeneic target cells pulsed or not with the OVA peptide (E) and values in each case were adjusted to control values (target cells not pulsed with the OVA peptide or medium stimulation, in the two assays respectively). Significance was determined with the student T-test where the * represents significance ($p < 0.05$) between OVA alone and α -GalCer + OVA and ¥ represents significance ($p < 0.05$) between α -GalCer + OVA 1 dose and α -GalCer + OVA 2 dose. This experiment was performed with 3 mice per group and repeated 3 times.

Fig. 4.2.3: Activation and expansion of NKT cells and DC after intranasal administration of a booster dose of α -GalCer

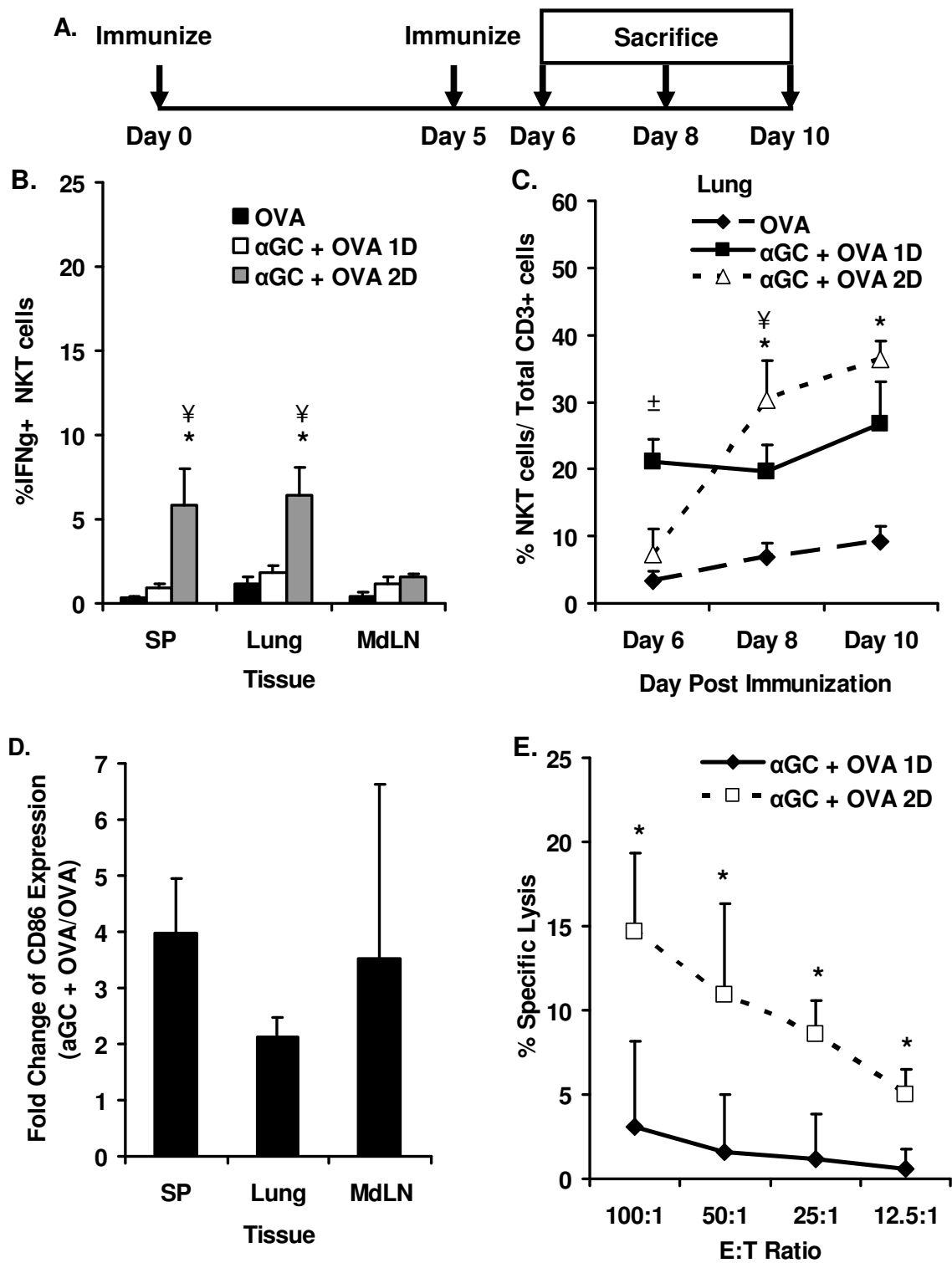


Fig. 4.2.3: Activation and expansion of NKT cells and DC after intranasal

administration of a booster dose of α -GalCer cont. To additional groups of mice immunized by the intranasal route with either OVA alone or admixed with α -GalCer, a booster dose was administered at day 23 after the primary immunization and the mice were sacrificed 24, 26, and 28 days which is 1, 3 and 5 days after the booster immunization (F). Live NKT+CD3+ cells producing IFN γ were enumerated in the spleen, lung and MdLN tissues on day 24 post-immunization and the average numbers with standard deviation values are calculated for the different groups of mice to determine statistically significant (G). The percentage of NKT tetramer+ cells from total CD3+ cells in the lung was measured at days 24, 26 and 28 post-immunization and significant differences between the groups are shown (H). The activation status of DC was assessed in terms of surface CD86 expression in cells as measured by flow cytometry from spleen, lung and MdLN on day 24 post-immunization and the fold change was calculated between groups (I). The antigen-specific IFN γ producing cells were assessed by the ELISPOT assay (J). Significance was determined with the student T-test where the * represents significance ($p < 0.05$) between OVA alone and α -GalCer + OVA and ¥ represents significance ($p < 0.05$) between α -GalCer + OVA 1 dose and α -GalCer + OVA 2 dose. This experiment was performed with 3 mice per group and repeated 3 times.

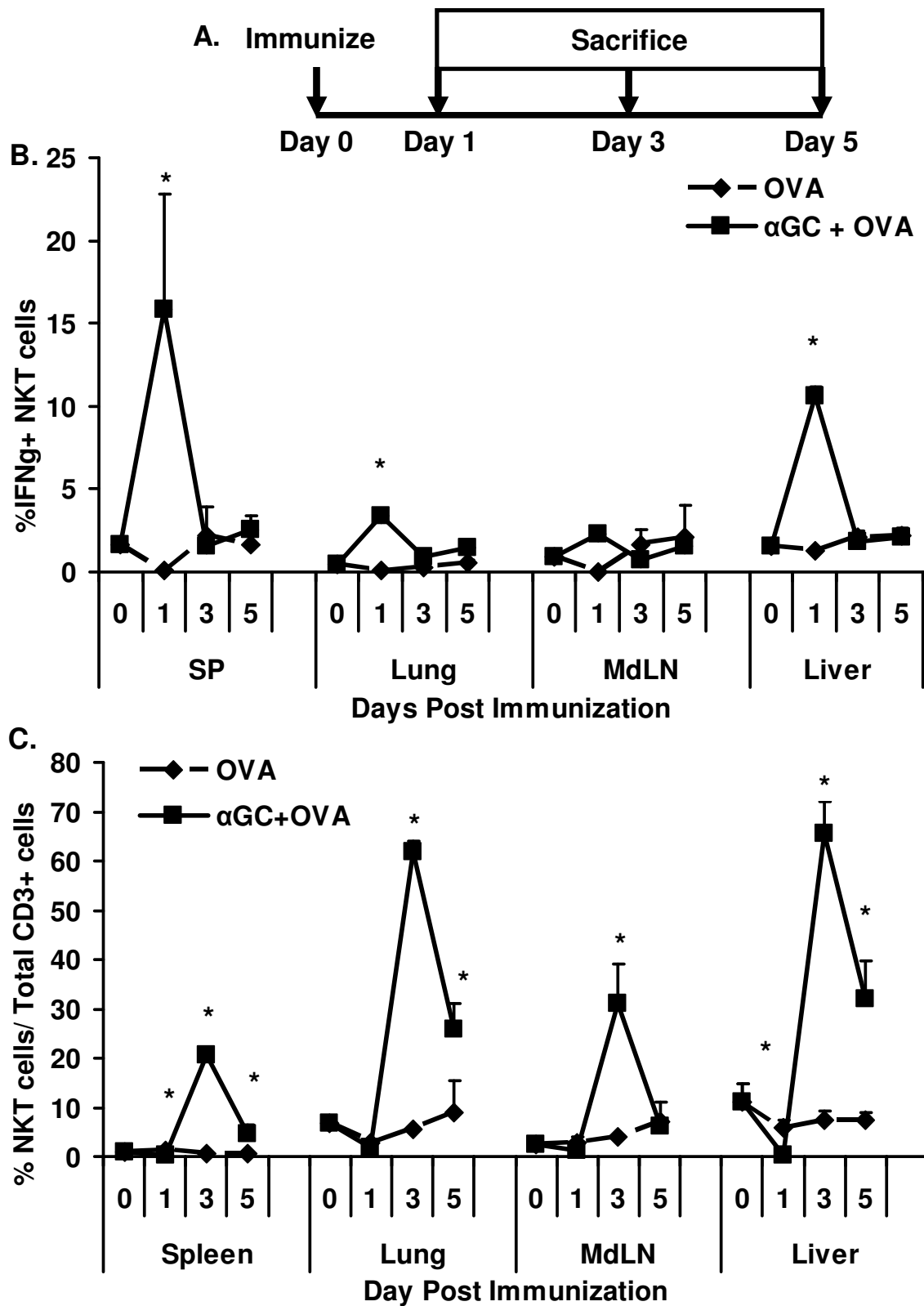
4.2.3. Intravenous but not intranasal immunization using α -GalCer adjuvant results in unresponsiveness of NKT cells to the booster immunization

Results from intranasal immunization shown above are in contrast to reports showing that a single dose of systemic α -GalCer administration either by the intravenous or intraperitoneal route leads to NKT cell anergy, which is defined as an unresponsiveness of NKT cells to a second or booster dose of α -GalCer administered by the same route, in terms of inability to produce IFN γ or proliferate (86, 101-102, 124). We investigated whether anergy versus reactivation of NKT cells is influenced by the route of α -GalCer administration (e.g. intranasal or intravenous). For this mice were immunized with OVA alone or OVA+ α -GalCer by the intravenous route (Fig. 4.2.4A) and tissues collected from mice sacrificed on days 1, 3 and 5 were analyzed for the phenotype and function of NKT cells and DC. Consistent with reports in the literature, significantly higher numbers of NKT cells producing IFN γ were observed along with NKT cell expansion in the spleen, lung and liver tissues of mice immunized intravenously with a primary dose of OVA mixed with α -GalCer compared to control group of mice that received OVA without α -GalCer (Fig. 4.2.4B and Fig. 4.2.4C). This is however different from what was observed in mice immunized by the intranasal route with OVA and α -GalCer, in that significantly lower levels ($p=0.006$) of IFN γ -producing NKT cells were observed in the lung of mice immunized by the intravenous route (compare Fig. 4.2.4B to 4.2.1C). Also, expansion of the NKT cells peaked at day 3 after intravenous immunization in all tissues tested and subsequently returned to baseline levels by day 5 (Fig. 4.2.4C), while a continued expansion of the NKT cells was observed in the lung until day 5 post-immunization and did not return to base level even at day 10 after immunization by the intranasal route (Fig. 4.2.1E).

Next, the effect of boosting with α -GalCer adjuvant delivered 23 days after the priming dose (the longer immunization scheme) was compared for the intravenous and intranasal routes of immunization (Fig. 4.2.4D and Fig. 4.2.3F, respectively). In mice receiving the booster immunization by the intravenous route, no significant increase in the IFN γ producing NKT cells was observed in the spleen, lung and liver tissues when compared to those in either the OVA alone control group or the group that received only a priming dose of α -GalCer along with OVA (Fig. 4.2.4E). This is in contrast to significant increases in the IFN γ producing cells observed in the spleen, lung and MdLN tissues of mice that received the booster dose of α -GalCer and OVA by the intranasal route at day 23 (Fig. 4.2.3G). Additionally, there was no expansion of NKT cells observed after delivering the intravenous booster immunization of α -GalCer + OVA, in any of the tissues tested (Fig. 4.2.4F), which also differs from the observations after intranasal booster immunization at day 23 (Fig. 4.2.3H). These data suggest that unresponsiveness of NKT cells, in terms of inability to produce IFN γ and proliferate, referred to as NKT cell anergy, may be a result of systemic versus mucosal administration of α -GalCer and the antigen.

Fig. 4.2.4: Activation and expansion of NKT cells after primary but not booster intravenous administration of α -GalCer. Mice were immunized by the intravenous route with a primary dose of OVA alone (OVA) or admixed with α -GalCer (α -GC + OVA) and sacrificed 1, 3 and 5 days post-immunization (A). Cells isolated from the spleen, lung, MdLN, and liver were stained with fluorescence labeled reagents: anti-CD3-PB, NKT tetramer-APC, anti-IFN γ -PE and Aqua live/dead stain. The live NKT+ CD3+ cells producing IFN γ were enumerated for each tissue between days 1 to 5 post-immunization for the OVA and α -GC + OVA groups of mice and significant differences were identified with * (B). The percentage of NKT tetramer+ cells from total CD3+ cells was measured to determine the kinetics of NKT cell expansion between days 1 to 5 post-immunization for the OVA and α -GC + OVA groups of mice and significant differences were identified with * (C). A booster dose of OVA alone or α -GC + OVA was administered to the respective group at day 23 after the primary immunization and the mice were sacrificed on days 24, 26, and 28 which is 1, 3 and 5 days after the booster immunization (D). Live NKT+CD3+ cells producing IFN γ (E) and the percentages of NKT tetramer+ cells from total CD3+ cells (F) were determined between days 24-28 post-immunization and the average numbers with standard deviation values are shown for each tissue. Significance was determined with the student's t-test where the * represents significance ($p < 0.05$) between groups that received OVA alone or α -GalCer + OVA. This experiment was performed with 3 mice per group and repeated once.

Fig. 4.2.4: Activation and expansion of NKT cells after primary but not booster
intravenous administration of α -GalCer



4.2.4. CD11c+ cells and not B220+ cells present α -GalCer in the lung and lung draining lymph node after intranasal administration of α -GalCer.

Next, we investigated whether the tissue origin and/or the phenotype of cells presenting the α -GalCer to the NKT cells would influence the anergy observed for NKT cells after intravenous versus intranasal route of administration. After intranasal immunization, cells isolated from the spleen, lung, and several mucosal draining lymph nodes of mice from either the α -GalCer group or OVA control group were co-cultured with the NKT cell clone (DN32.D2), and IL-2 production in the culture supernatants was assessed as a measure of α -GalCer presentation by cells isolated from the various tissues as described in the literature (125). We observed that cells from the lung and the lung draining lymph nodes (MdLN) on day 1 post-immunization activated the NKT cell clone suggesting that these two tissues to be effective in presenting α -GalCer (Fig. 4.2.5A). This activity (α -GalCer presentation) decreased in the lung significantly between days 1 and 5, but remained stable in the MdLN through day 5. None of the other tissues tested were able to activate the NKT cell clone. These results suggest that α -GalCer is presented mainly in the lung and lung draining lymph nodes after intranasal immunization. These results together with the data showing NKT cell activation/expansion data above (Figs. 4.2.1-4.2.3), suggest that the lung is the major responding tissue after intranasal immunization employing α -GalCer as the adjuvant.

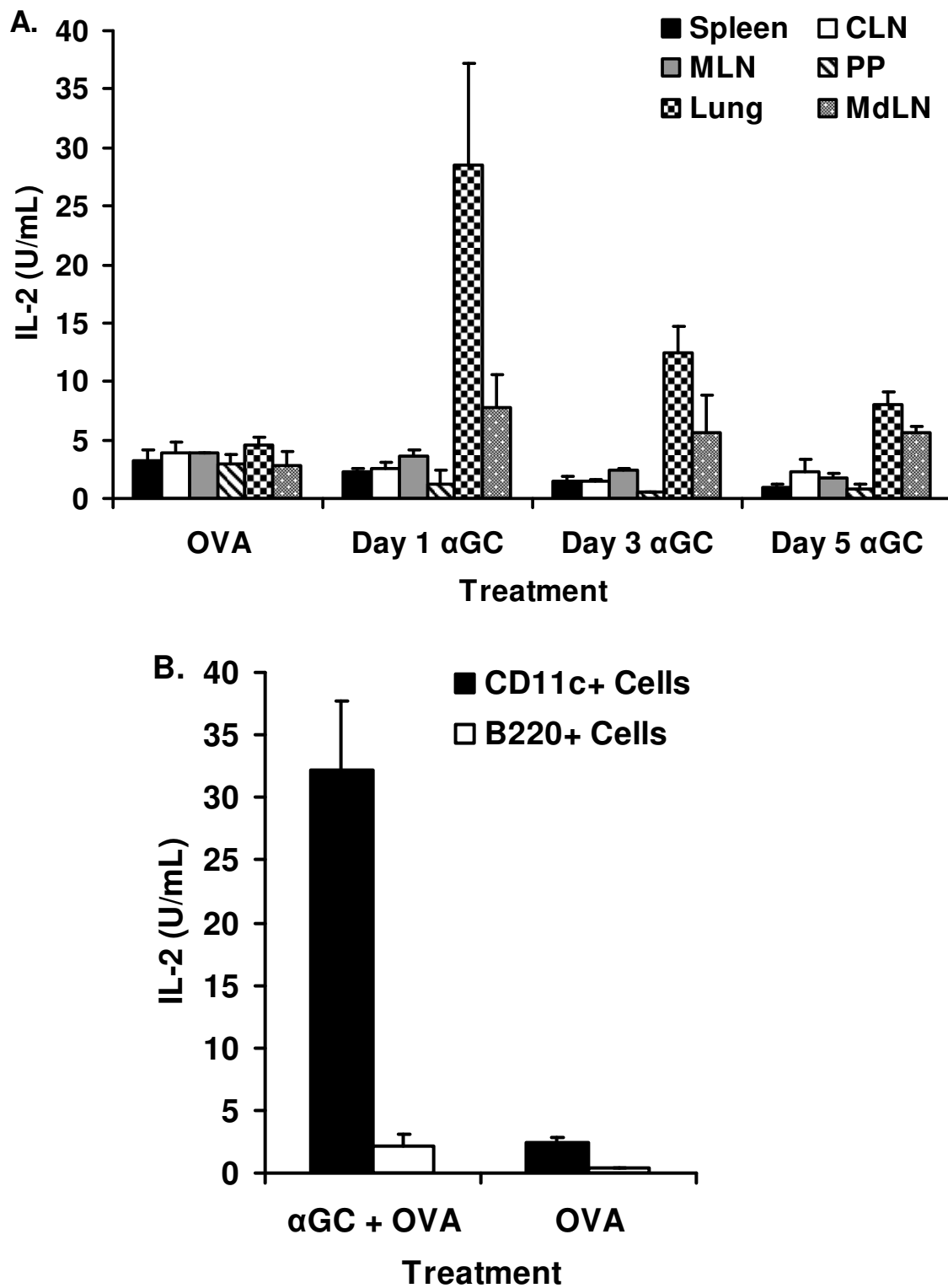
The phenotype of the α -GalCer presenting cells in the lung on day 1 after intranasal immunization with α -GalCer + OVA was further investigated by purification of the CD11c+ (DC) or B220+ (B cells) populations using the MACs bead separation procedure. Subsequently, the purified cells were co-cultured with the NKT cell clone (DN32.D2), and the supernatants were analyzed for IL-2 production. In the lungs of

mice from the α -GalCer group, only the CD11c⁺ cells, but not B220⁺ cells, induced IL-2 production while neither cell type from lungs of mice immunized with OVA alone activated the NKT cell clone (Fig. 4.2.5B). This data suggests that most likely DC and not B cells are involved in selectively presenting α -GalCer to NKT cells in the lung after intranasal administration of α -GalCer. This is in contrast to reports in the literature that showed α -GalCer presentation by the B cells in mice immunized by the intravenous route, which was hypothesized to be a major reason for the anergy of NKT cells (86, 103, 125).

Fig. 4.2.5: Tissue and cell type for α -GalCer presentation after intranasal

immunization. Mice were immunized by the intranasal route with either OVA alone or admixed with α -GalCer (α -GC + OVA) and sacrificed on days 1, 3 and 5 post immunization. Cells isolated from the spleen, lung, MdLN along with cervical lymph nodes (CLN), mesenteric lymph nodes (MLN), and peyer's patches (PP) as cells potentially capable of presenting α -GalCer were co-cultured with the NKT cell clone DN32.D2. The supernatants from the co-cultures were analyzed for IL-2 production (A). Separate groups of mice were immunized by the intranasal route with OVA alone or admixed with α -GalCer (α -GC + OVA) and sacrificed on day 1 post immunization. The cells were then stained for either CD11c-PE or B220-PE and purified by MACs sorting with PE MACs beads. The isolated CD11c+ and B220+ cells were co-cultured with the NKT cell clone DN32.D2 and the culture supernatants were analyzed for IL-2 production (B). This experiment was performed with 3 mice per and repeated twice.

Fig. 4.2.5: Tissue and cell type for α -GalCer presentation after intranasal immunization



4.2.5. Evaluation of PD-1 expression on NKT cells after intranasal immunization with α -GalCer adjuvant.

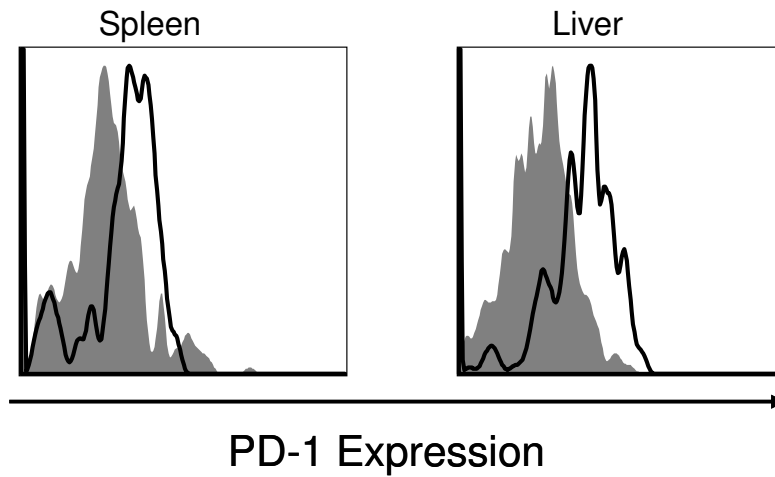
Recent reports in the literature implicated increased PD-1 expression in NKT cells for the observed anergy resulting from the administration of α -GalCer by the systemic routes (104-105, 126). To test this, NKT cells from different tissues of mice immunized either by the intravenous or intranasal route with α -GalCer + OVA were examined for PD-1 expression. Significantly higher PD-1 levels were observed on the NKT cells from spleen (3.7 fold and $p=0.019$) and liver (11.5 fold and $p=0.0016$) of mice at day 1 after immunization with α -GalCer and OVA by the intravenous route when compared to NKT cells from mice immunized with OVA alone (Fig. 4.2.6A and 4.2.6C). However, after intranasal immunization PD-1 levels on the NKT cells from spleen and lung tissues of mice from the α -GalCer group were not similarly increased when compared to cells from mice in the OVA alone group (Figs. 4.2.6B and 4.2.6C). We also did not observe increased PD-1 levels on cells from mice receiving a booster dose of α -GalCer + OVA on day 5 post-primary immunization (i.e. two doses of α -GalCer total). Thus, NKT cells in the lungs of mice immunized by the intranasal route using α -GalCer as an adjuvant exhibit no changes in the PD-1 expression on day one post immunization and no signs of functional anergy, in terms of cytokine production and expansion. These results support the hypothesis that mucosal, as opposed to systemic administration of α -GalCer (i.e. intranasal versus intravenous route) may lead to different consequences for NKT cells in terms of induction of anergy or functional competence in response to repeated α -GalCer delivery.

Fig. 4.2.6: PD-1 expression on NKT cells after intravenous or intranasal

administration of α -GalCer. Mice were immunized by the intravenous or intranasal route with either OVA alone (OVA) or admixed with α -GalCer (α -GC + OVA) and sacrificed on day 1 post-immunization. Cells isolated from the spleen and lung or liver were stained with fluorescence labeled reagents: anti-CD3-PB, NKT tetramer-APC, anti-PD-1-FITC and Aqua live/dead stain and analyzed by flow cytometry. The live NKT+ CD3+ cells expressing PD-1 were enumerated and representative histograms show data for the spleen and liver tissues from mice immunized by the intravenous route (A) or from spleen and lung tissues of mice immunized by the intranasal route (B) with either OVA alone (filled histogram) or α -GC + OVA (open histogram). Panel C shows the average data with standard deviation values for the fold difference between the OVA and α -GC + OVA groups of mice for the expression of PD-1 on NKT cells at day 1 post-primary immunization by the intravenous (IV 1 Dose) and intranasal (IN 1 Dose) routes or after booster immunization by the intranasal route (IN 2 Dose). This experiment was performed with 3 mice per group and repeated three times.

Fig. 4.2.6: PD-1 expression on NKT cells after intravenous or intranasal administration of α -GalCer

A. IV Immunization – Day 1 post immunization



B. IN Immunization – Day 1 post immunization

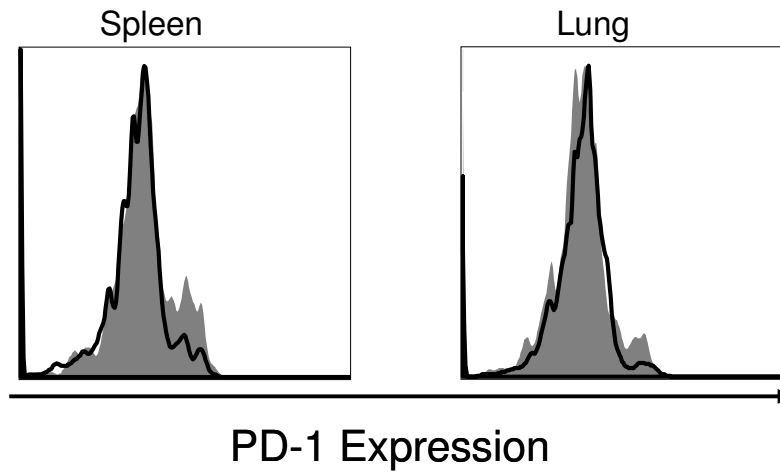
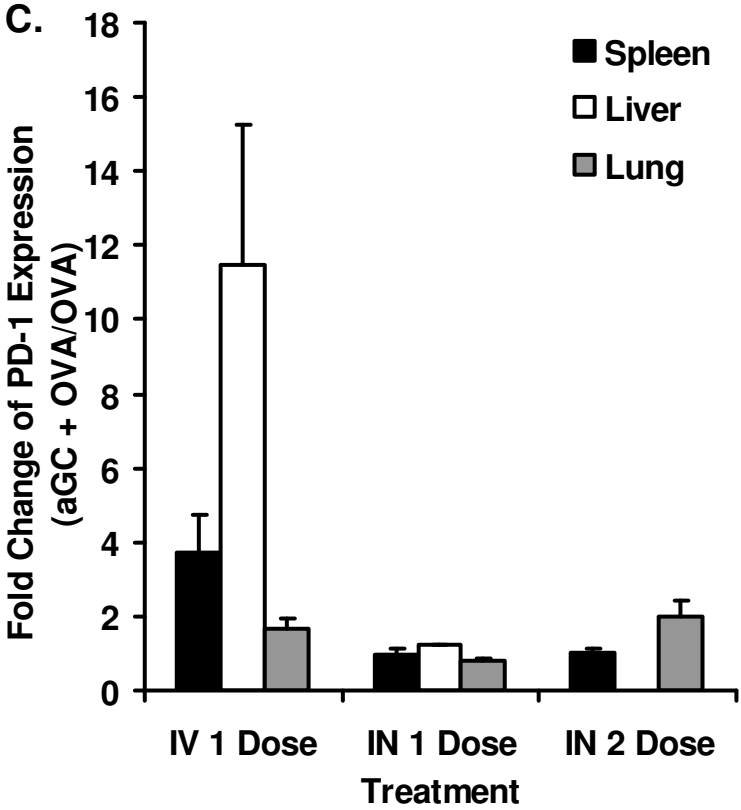


Fig. 4.2.6: PD-1 expression on NKT cells after intravenous or intranasal administration of α -GalCer



CHAPTER 5

DISCUSSION

Results from this investigation provide the first evidence that mucosal delivery is an effective means to harness the adjuvant potential of α -GalCer for vaccination regimens which deliver multiple doses to achieve strong cellular immune responses to co-administered immunogens. Unlike systemic vaccination where delivering a single dose of α -GalCer leads to anergy of responding NKT cells and thus hinders delivery of booster immunizations (86, 101-102), we demonstrated that administration of multiple doses of α -GalCer by the intranasal or oral route affords repeated activation of NKT cells and the induction of broad systemic and mucosal immunity. This is specifically advantageous, and may be even essential, for vaccination regimens against mucosal pathogens such as the human immunodeficiency virus (HIV) and the human papillomavirus (HPV), where priming of durable protective immunity at the mucosal portals of pathogen entry would be highly desirable. Currently alum is the only adjuvant approved by the Food and Drug Administration (FDA) and only for systemic vaccination approaches (54, 64). Evidence from the present investigation strongly suggests that α -GalCer, a synthetic lipid with a proven safety profile established in multiple human clinical studies (87, 98-100, 109), is a promising candidate mucosal adjuvant for inclusion in vaccine formulations against a wide range of pathogen-mediated diseases as well as cancers.

The importance and advantage of a multiple-dose immunization scheme delivering α -GalCer was realized in the studies employing this adjuvant admixed with an HIV envelope peptide, R15K, to vaccinate mice by the intranasal or oral route where two or three doses of adjuvant-antigen mixture resulted in the induction of strong peptide-

specific cell-mediated immunity in systemic and mucosal tissues, including the more distal mucosal sites such as the mesenteric lymph nodes (MLN) in the gut (96). Induction of strong antigen-specific immune responses in the gut is particularly important for protection against HIV because during primary infection, a majority of the host cell targets for viral infection, replication and persistence are located in the intestinal mucosal tissues. Furthermore, in primary HIV infection as well as SIV infection in macaque models severe depletion of CD4⁺ T-cells in the gut associated lymphoid tissues (GALT) coincided with high viral loads, while patients that maintain normal levels of CD4⁺ T-cells in the GALT correlate with undetectable viral loads and stable CD4⁺ T-cells in the blood(127-129). It is therefore believed that HIV immunization strategies should target these tissues to stimulate effector cells for eliminating viral persistence (130). Thus, our studies showing the efficiency of α -GalCer for inducing antigen-specific effector T-cells in the gut mucosal tissues, specifically the MLN, strongly support α -GalCer as an attractive candidate adjuvant for usage in HIV vaccine formulations.

In addition to the route, the immunization schedule employed in our studies was also observed to play an important role in the priming of antigen-specific gut-mucosal immunity. Mice immunized with one or more booster doses, relative to those that received only the primary dose of the mixture of α -GalCer and HIV peptide by either intranasal or oral route were able to develop strong antigen-specific systemic and mucosal immune responses in intestinal mucosal sites. These results suggest that administration of booster doses of free α -GalCer by the intranasal or oral route is necessary to induce antigen-specific T-cell responses in the various mucosal tissues. These results confirm and extend similar studies by Kang (123) where three intranasal doses of α -GalCer ad-mixed with either the OVA protein or the HA protein from the

influenza virus were effective in priming strong antigen-specific systemic and mucosal immune responses.

Several lines of evidence from the present investigation provide mechanistic explanation for the adjuvant potential of α -GalCer delivered by the intranasal route. First, we observed strong activation of NKT cells and DC in the lungs of mice after both the primary and the booster intranasal immunization with α -GalCer admixed with OVA antigen, while systemic immunization by the intravenous route resulted in the unresponsiveness of the NKT cells to booster doses of α -GalCer, a phenomenon referred to in the literature as NKT cell anergy (86, 101-102). These results are consistent with the data from our studies showing repeated immunization with α -GalCer via mucosal routes to be beneficial and necessary for the induction of broad antigen-specific systemic and mucosal cellular immune responses (96). To our knowledge, this is the first time that an immunization route for delivering α -GalCer has been implicated as a potential cause for the induction (or the lack) of NKT cell anergy.

While a comparison of a single intravenous and intranasal route of α -GalCer delivery for the kinetics of activation of NKT cells and DC revealed similar results in terms of peak levels of IFN γ production by NKT cells and DC activation at one day after immunization, booster immunization by the two different routes revealed vastly different effects on NKT cells and DC. Our results for intravenous administration of α -GalCer support reports in the literature in that after a single administration of α -GalCer, NKT cells become unresponsive, unable to produce cytokines in response to a booster dose of α -GalCer and also unable to proliferate. (86, 101-102) On the other hand, booster intranasal administration of α -GalCer demonstrated potent re-activation of the NKT cells in the lung, including IFN γ -production and expansion, and DC activation. This re-

activation of NKT cells and DC occurred regardless of the timing of the booster dose (i.e. day 5 versus 23), suggesting that immunization by the intranasal route is a potential means to avoid the induction of NKT cell anergy and allow repeated dosing with α -GalCer which is important for inducing broad systemic and mucosal antigen-specific immunity. A recently published report has shown that delivery of α -GalCer by the intradermal route is also effective in avoiding NKT cell anergy, but mechanistic details are not described (131).

Another important finding from the present investigation is that after intranasal administration, CD11c+ cells more efficiently presented α -GalCer in the lung when compared to B220+ cells, suggesting that the intranasal immunization route preferentially targets α -GalCer presentation to CD11c+ cells (DC). These results support and extend literature reports showing administration of B cells, but not DC, pulsed ex vivo with α -GalCer to induce NKT cell anergy (86, 101, 103). Collectively, these reports in the literature along with data from our investigation suggest that the specific cell type that initially presents α -GalCer plays an important role in the induction of NKT cell anergy and the potential of mucosal delivery to target α -GalCer to these cells is important to avoid induction of anergy to NKT cells.

Finally, we observed that while NKT cells from mice administered with α -GalCer by the intravenous route exhibited high levels of the cell surface marker programmed death -1 (PD-1), a marker associated with functional exhaustion of effector CD4, CD8 and NK subsets of T cells (104-108, 126, 132), those in mice where α -GalCer is delivered by the intranasal route did not. Thus, in addition to the cell type mediating α -GalCer presentation, the phenotype of NKT cells in terms of PD-1 expression, could be another important difference resulting from mucosal α -GalCer delivery (e.g. intranasal route), as

opposed to systemic delivery (e.g. intravenous route). These differences observed for the delivery of α -GalCer by the intranasal route may be the potential reason for efficient re-activation of NKT cells, which aids in promoting DC activation and allows α -GalCer to serve as an efficient adjuvant to induce immune responses to co-administered antigens.

In addition to the differences in terms of NKT cell anergy induction, there were several other differences observed for NKT cell activation after intravenous versus intranasal administration of α -GalCer. First, the timing of NKT cell activation and expansion appeared to be prolonged after intranasal administration of α -GalCer because the peak levels of NKT cell expansion were observed at day 5 and stayed relatively at high levels till day 10 post immunization in the lung, the main responding tissue for this route of immunization. These results differ from that seen after the intravenous immunization where the NKT cell population peaked at day 3 and returned to base levels soon after in all tissues tested. In this regard, it is important to note that Fujii et al (86), who reported that intravenous administration of DC pulsed ex vivo with α -GalCer, as opposed to free α -GalCer, will not cause anergy to NKT cells, also showed that α -GalCer-pulsed DC prolonged NKT cell response, as measured by IFN γ production, when compared to free α -GalCer. Second, we observed a decrease in the NKT cell population in the spleen and liver at day 1 after the priming immunization by the intravenous route, which is consistent with literature reports that attribute the decrease in population to the down-regulation of the TCR as the underlying mechanism, but no such decrease in NKT cells was observed in mice that received the priming immunization by the intranasal route. Incidentally, Fujii et al (86) reported a phenomenon describing NKT cell turnover (a decrease in the NKT cell population on day 1 after α -GalCer administration later found to be due to TCR down-regulation) after administration of free α -GalCer that was “less rapid and severe” when DC pulsed with α -GalCer were administered.

Overall, this study has shown that α -GalCer as an adjuvant can be administered by the intranasal route for primary and booster immunizations to induce cellular immune responses to co-administered antigens, without inducing anergy to NKT cells because of: (a) potential presentation of α -GalCer by DC, (b) prolonged activation of NKT cells after primary immunization and re-activation after booster immunization, in the lungs, and (c) no increase in the expression of inhibitory PD-1 molecules. Thus mucosal immunization by the intranasal or oral route may be an attractive strategy for immunization because it affords the ability to deliver multiple doses of the vaccine, which is important for most therapeutic applications against infectious diseases and cancer.

CHAPTER 6

SUMMARY AND CONCLUSIONS

This study identified mucosal delivery of α -GalCer as an important means to avoiding anergy to NKT cells and showed that efficient reactivation of NKT cells with multiple doses of α -GalCer is the mechanism of adjuvant activity of α -GalCer that induces broad systemic and mucosal cellular immune responses to co-administered antigens. After intranasal immunization, α -GalCer presentation by CD11c⁺ cells led to activation and expansion of NKT cells which occurred predominantly in the lungs and the lung-draining mediastinal lymph nodes (MdLN), the latter tissue also exhibited DC activation and efficient presentation of co-administered antigen to T cells (adaptive immune responses). Based on the experimental evidence obtained in this investigation, combined with literature reports, we propose a model depicting the superior adjuvant potential of α -GalCer delivered by the intranasal route relative to that after intravenous administration. This model shows activation of NKT cells after the priming dose of the intranasal immunization mediated by the presentation of α -GalCer by CD11c⁺ cells (most likely DC), which in turn leads to activation of the DC in the lung (Fig. 6.1.1A). In this model we suggest that subsequent to activation, the NKT cells and/or DC potentially may migrate to the lung-draining MdLN and then eventually to spleen, because activated NKT cells and DC were observed in these tissues. However, based on studies employing the α -GalCer adjuvant along with the HIV peptide R15K (Fig. 4.1.2C) or OVA protein (Fig. 4.2.2C), antigen presentation by activated DC occurred mainly in the MdLN, and consequently, antigen-specific immune responses were only detected in the MdLN after primary intranasal immunization. In comparison to this, after primary intravenous immunization with the α -GalCer adjuvant along with OVA protein, the liver has been identified as the major responding tissue, where α -GalCer is presented by multiple APCs

including DC and B cells (86). We (Fig. 4.2.4) and others (86) have also detected higher numbers of activated NKT cells and DC at this site and these details are summarized as shown in the model (Fig. 6.1.1B). Incorporating the results obtained in the present investigation, the model further shows the consequence of booster immunization with α -GalCer by the intranasal route in terms of re-activation of both the NKT cells and DC in the lung, MdLN and spleen (Fig. 6.1.1C). In support of this model, we observed antigen-specific immune responses in the lung, MdLN and spleen after one or two booster immunizations with the mixture of α -GalCer and the HIV peptide R15K administered by the intranasal route (Fig. 4.1.3). This is in sharp contrast to what was observed after a booster intravenous immunization with α -GalCer mixed with OVA protein, where no activation of NKT cells or DC was observed (Fig. 4.2.4), and these contrasting results compared to those after intranasal booster immunization, are incorporated in the model (Fig. 6.1.1D).

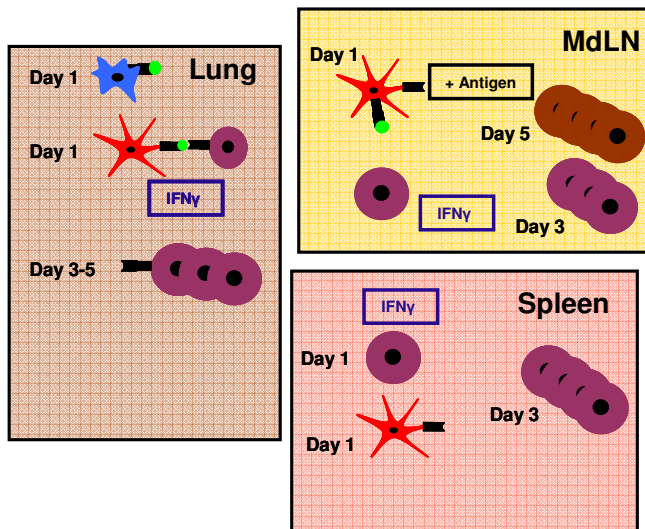
In summary, our model incorporating the results obtained in this study describes the potential mechanism for efficient adjuvant activity of α -GalCer administered by the intranasal route, in that the NKT cells can be re-activated after intranasal immunization with α -GalCer, and this re-activation is the potential mechanistic basis for the observed broad antigen-specific systemic and mucosal immune responses to the co-administered antigens. Therefore, immunization with the adjuvant α -GalCer, by the intranasal route, is a promising vaccination scheme, because multiple doses of the adjuvant can be administered without inducing NKT cell anergy, and the ability to administer multiple doses is important for most therapeutic applications against infectious diseases and cancer.

Figure 6.1.1: Model of α -GalCer mediated activation of immune cells after intranasal vs. intravenous administration. This model represents the responses of the different immune cells after either a primary or booster dose of α -GalCer by the intranasal or intravenous immunization route. Panel A illustrates changes in the activation/proliferation of immune cells in the lung, MdLN and spleen after a single intranasal dose of α -GalCer. On day 1 post-immunization with α -GalCer+OVA, α -GalCer is presented by DC to the NKT cells of the lung, and NKT cells and DC are subsequently activated in the lung, MdLN and spleen on day 1. Additionally, NKT cells proliferate mainly in the lung and spleen between days 3 to 5, and antigen specific immune responses are observed in the MdLN on day 5 post immunization. Panel B illustrates responses of immune cells in the liver and spleen after intravenous immunization with a single dose of α -GalCer+OVA. Based on literature reports it is shown that α -GalCer is presented by both DC and B cells in the liver and the spleen, leading to activation of NKT cells and DC, and antigen-specific immune responses in the spleen. Panel C depicts the changes to the immune cells after a booster immunization with α -GalCer+OVA by the intranasal route. Presentation of α -GalCer occurs in the lung leading to activation of the NKT cells and DC in the lung, MdLN and spleen. However, complete activation of NKT cells occurs mainly in the lung, where NKT cell proliferation is observed days 3 to 5 after booster immunization. Additionally, antigen specific immune responses are detected in the lung, MdLN and spleen after the booster immunization. Finally, panel D depicts the effects of a booster immunization with α -GalCer+OVA by the intravenous route, where, although α -GalCer presentation is most likely occurring, no activation of NKT cells or DC occurs in the liver or spleen.

Figure 6.1.1: Model of α -GalCer mediated activation of immune cells after intranasal vs. intravenous administration.

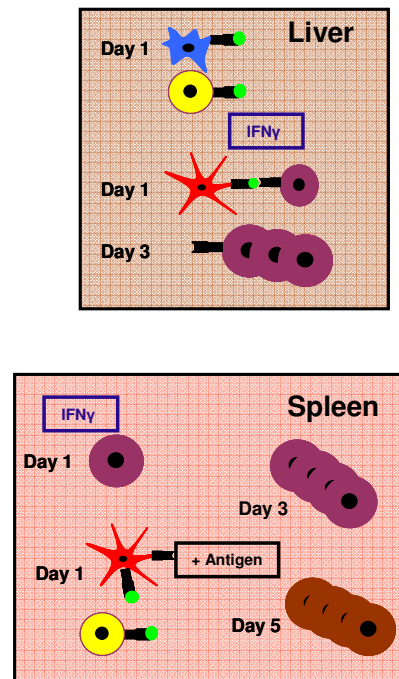
A.

Intranasal Administration of α -GalCer – Primary Dose



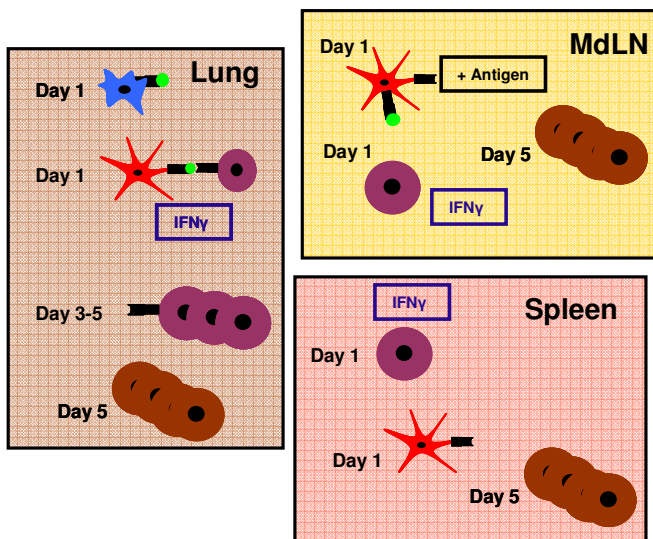
B.

Intravenous Administration of α -GalCer – Primary Dose



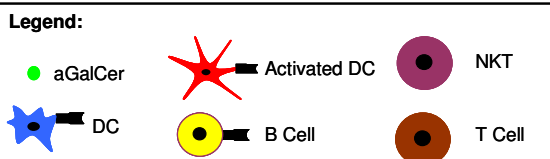
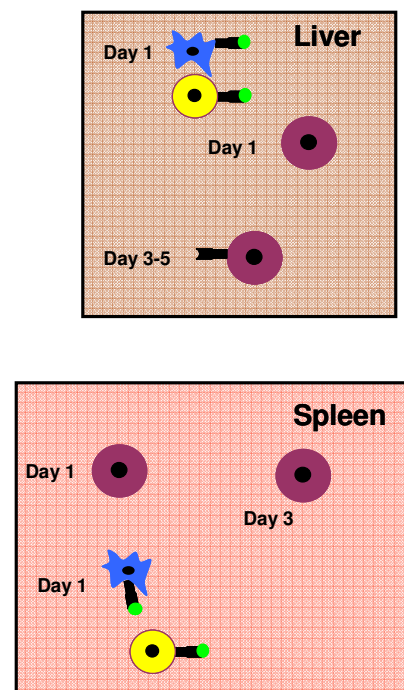
C.

Intranasal Administration of α -GalCer – Booster Dose



D.

Intravenous Administration of α -GalCer – Booster Dose



CHAPTER 7

FUTURE DIRECTIONS:

An important aspect of this investigation is the demonstration of the effectiveness of the adjuvant potential of α -GalCer, specifically for the mucosal vaccination approaches. This is based on the data showing that: (a) repeated doses of α -GalCer may be delivered by the mucosal route to induce strong mucosal and systemic immune responses to the co-delivered antigen; (b) re-activation of NKT cells and DC occurs with each round of intranasal α -GalCer administration; and (c) α -GalCer can be administered by the intranasal route without the undue effects of anergy for NKT cells that otherwise result from systemic delivery of α -GalCer.

7.1. ALTERNATIVE α -GALCER IMMUNIZATION FORMULATIONS

While data from this investigation highlight the advantage of repeated intranasal delivery of α -GalCer for NKT cell-mediated activation of DC and efficient antigen presentation, there is some concern in the literature based on observations that described increased numbers of NKT cells in the lungs to adversely influence conditions of asthma in patients (133-135). However, other studies reported no such increase or adverse effects after intranasal α -GalCer delivery (135-138). Nevertheless, our studies also showed the effectiveness of oral immunization employing multiple doses of α -GalCer adjuvant to induce systemic and mucosal immune responses to co-administered antigens (96). Therefore, detailed analyses of the kinetics of activation and expansion of NKT cells along with that of DC in various tissues following oral immunization would substantiate the suitability of this route as an alternate to intranasal immunization for efficient delivery of vaccine formulations employing α -GalCer adjuvant. In addition to investigating the

kinetics of activation of NKT cells and DC after oral immunization with the α -GalCer adjuvant, it would also be important to investigate the phenotypes and subsets of DC that are activated in response to NKT cell activation in order to determine whether these phenotypes influence the strength and breadth of antigen-specific immunity. In this regard, it has been shown that different subsets of DC that express certain cell surface markers, e.g. DEC205, CD103, or chemokine receptors, e.g. CCR6 and CX3CR1, as well as the specific location within the gut-associated lymphoid tissues effects the ability of the DC to either more efficiently present antigen and activate lymphocytes or to maintain the tolerogenic environment of the gut (23, 139-142).

One important aspect of our investigation that needs specific consideration is the observation that the levels of PD-1 expression on NKT cells in mice do not increase after immunization with α -GalCer by the nasal route. Thus our investigation implies that lower levels of PD-1 expression on NKT cells are associated with overcoming anergy and therefore allow repeated administration to achieve enhanced immune responses to co-administered antigens. In accordance with this interpretation literature reports showed that blockage of the PD-1/PD-L1 interaction may lead to re-activation of T cells and NKT cells (104-107). However, the down-regulation of PD-1 has also been shown to play a role in various autoimmune diseases, as reviewed in Okazaki T et al (143). PD-1 knockout mice have been shown to develop various autoimmune diseases depending on the genetic background of the mice. For example, Balb/C mice that have a disruption in the PD-1 gene develop dilated cardiomyopathy and an increase in auto-antibodies to cardiomyocytes (144). Additionally, C57Bl/6 mice with the PD-1 knockout mutation develop lupus-like proliferative arthritis and glomerulonephritis, as measured by histology and immunohistochemistry (145). In agreement with these murine model findings, single nucleotide polymorphisms of PD-1 have been identified in several human

autoimmune diseases, including systemic lupus erythematosus (146), type I diabetes (147), rheumatoid arthritis (148-149), and celiac disease (150-151). Since there is an apparent decrease in PD-1 expression on NKT cells after mucosal delivery of α -GalCer, then immunization with α -GalCer may induce autoimmune responses. However, literature search of α -GalCer delivery by systemic routes reveals that α -GalCer has actually been utilized as a treatment for certain autoimmune diseases, although the role of α -GalCer is controversial (152). For example, the α -GalCer stimulation of NKT cells has been shown to be both beneficial (153-154) and harmful (155) in various rheumatoid arthritis models. Nevertheless, the effect of an α -GalCer mediated decrease in PD-1 expression needs to be further examined to determine whether immunization with this adjuvant can induce certain autoimmunity responses. First, it would be important to determine whether the decreased PD-1 expression on NKT cells is transient, or whether it is a permanent down-regulation of the receptor. Additionally, the expression of PD-1 should be evaluated on other cell types in addition to NKT cells, such as T and B cells after both oral and intranasal administration of α -GalCer. Finally, mice immunized with the α -GalCer adjuvant should be assessed for signs of autoimmunity.

Since induction of anergy to NKT cells has been recognized as the major obstacle in fully realizing the potential clinical utility of α -GalCer (156), several alternate strategies have been explored including designing analogs of α -GalCer and liposome and nanoparticle formulations to substitute for free α -GalCer. Systemic administration of different analogs and formulations of α -GalCer, as opposed to free α -GalCer, have been shown to reduce or avoid anergy to NKT cells. For example, it was shown that OCH, a synthetic glycolipid analog of α -GalCer with a truncated sphingosine chain (157-158), was reported to not induce NKT cell anergy to the extent that α -GalCer does. OCH also was shown to not induce the expression of the cell surface marker Qa-1^b, which is found

on APC and has been implicated in the induction of anergy of NKT cells, to the extent that α -GalCer induces Qa-1^b expression (124). More recently, a poly-lactic acid based nanoparticle formulation of α -GalCer was reported to stimulate NKT cells without inducing anergy after intravenous injection (120). In this study it was shown that the nanoparticle formulation of α -GalCer was efficiently presented by CD11c+ cells relative to B220+ cells, a mechanism similar to the one described in our studies, where the α -GalCer administered by the intranasal route was selectively targeted to CD11c+ cells (DC). It would be advantageous to compare these analogs/formulations of α -GalCer to free α -GalCer to determine if they would be suitable for mucosal administration and determine the optimal combinations of the adjuvant formulation and route for designing a vaccination regimen to induce efficient antigen-specific immunity.

7.2. IDENTIFICATION OF ADDITIONAL NKT CELL ANERGY AND NKT CELL ACTIVATION MARKERS

One important observation in this investigation pertaining to the adjuvant potential of α -GalCer is the advantage of delivery by the intranasal route to avoid NKT cell anergy determined in terms of the lower levels of PD-1 surface marker expression. While PD-1 expression has been documented as a potential marker for functional exhaustion of different T cell subsets, it is also possible that variations in the expression of other phenotypic and functional markers may contribute differentially for not only anergy or the lack thereof, but also for selective activation of NKT cells resulting after mucosal α -GalCer administration. For example, several inhibitory receptors have been examined by other labs with differing results, including the Qa-1^b ligand NKG2A and CTLA-4 (102, 124). A systems-based approach in terms of comparative microarray analysis of NKT cells from the major responding tissues after intravenous versus intranasal immunization (e.g. lung and liver, respectively) would be most beneficial to identify potential markers

that modulate activation versus anergy in the NKT cells. Such analyses would evaluate NKT cells obtained at several different time-points after delivering the α -GalCer, since the up- or down-regulation of surface markers may occur at different time-points relative to the α -GalCer stimulation. For example, it would be ideal to examine NKT cells at 24 hours post immunization, when maximal NKT cell activation is occurring and cytokines are being produced at peak levels. In addition, NKT cells should be examined at a time-point sometime between days 3 to 5 when the NKT cell expansion is occurring. Another time point of importance would be days 7 to 10 post immunization when the NKT cell population is contracting.

These microarray analyses would provide invaluable data for gene expression of NKT cells at different stages of NKT cell activation, and provide a better idea of which markers to examine in more detail on not only NKT cells, but it would also provide an idea of which associated ligands on APC to study as well. This microarray data would also provide a better picture of how NKT cell anergy is occurring in this system and how APC are able to modulate the anergy phenotype. The potential expression of NKT activation markers, transcription factors, cytokine/chemokines, migration markers, etc can also be explored to determine other potential modulators that influence the efficient induction of antigen-specific immune responses after immunization by these routes. Eventually data from such analyses could be utilized in the design of new vaccine formulations, to either actively inhibit or induce the identified important markers so that vaccines could be administered in multiple doses to elicit stronger antigen-specific immune responses.

CHAPTER 8

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CHAPTER 9

VITA

Amy Nicole Courtney was born in Washington Iowa on January 16th 1982 to John and Ellen Courtney. After graduating from Lewisville High School in Lewisville Texas in 1999, she went on to pursue a Bachelor of Science in Biology at Texas A&M University in College Station Texas, which she completed in May 2003. In August of 2003, she entered the PhD degree program at The University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences. In June 2006 she began her PhD work with Dr. K. Jagannadha Sastry at the University of Texas M.D. Anderson Cancer Center Department of Immunology.

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